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An investigation into the consequences of *Salmonella* infection of murine macrophages and dendritic cells: relevance to reactive arthritis

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Abstract

Reactive arthritis (ReA) is a sterile joint inflammation triggered by bacterial infection and is associated with the MHC class I molecule HLA-B27. Bacterial antigens have been detected in synovial tissues from ReA patients, but how they are transported to the joints from the site of infection is not known. In this project, we have tested the role of infected macrophages and dendritic cells as carriers of bacterial antigens in a mouse model for ReA.

Bone marrow derived macrophages (BMMac) and dendritic cells (BMDC) were infected with *Salmonella*, a known causative agent of ReA, and adoptively transferred to HLA-B27 transgenic mice. We found a differential migration profile for *Salmonella* infected macrophages vs dendritic cells. In contrast to their uninfected counterparts, both populations emigrated significantly from the peritoneal cavity after intraperitoneal (i.p.) injection. *Salmonella*-infected BMDC showed a significantly increased ability to migrate to secondary lymphoid organs (SLO) (spleen and mesenteric lymph nodes). Moreover, results from CCR7 deficient mouse clearly showed that the migration of infected BMDC to the mesenteric lymph nodes (MLN), but not the spleen, was mediated by CCR7. Unlike BMDC, *Salmonella* infected BMMac were prone to migrate to inflamed joints.

Although both BMDC and BMMac were able to present bacterial-derived peptides and stimulate antigen experienced cytotoxic T cells *in vitro*, only BMDC were capable of activating antigen specific naive T cells. By co-transfer HLA-B27 transgenic BMDC and naive T cells from GRb transgenic mice, which

express a HLA-B27–restricted TCR specific for an influenza peptide, BMDC infected with recombinant *Salmonella* expressing the flu epitope were shown to induce T cells activation *in vivo*.

The results of this study suggest distinct roles for macrophages and dendritic cells as antigen transporters following *Salmonella* infection, and have implications for both the pathogenesis of ReA, and the generation of anti-*Salmonella* immunity.

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Abbreviations

AEC	3-amino-9-ethylcarbazole
APC	Allophycocyanin
Ab	Antibody
ALN	Axillary lymph node
APCs	Antigen-presenting cells
β_2m	beta2-microglobulin
β-gal	β-galactosidase
BM	Bone marrow
BMDC	Bone marrow derived dendritic cells
BMMac	Bone marrow derived mcrophages
bp	Base pair
BSA	Bovine Serum Albumin
C5 (TS)	Temperature sensitive strain C5
CCD	Cytochalasin D
cDNA	Complementary DNA
CFSE	Carboxy-fluorescein diacetate, succinimidyl ester
CFU	Colony-forming unit
CMTMR	(5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine
CR3	Complement receptor 3
CTL	Cytotoxic T lymphocyte line
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
EBI3	Epstein Barr virus induced protein 3
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot assay
ER	Endoplasmic reticulum
FACS	Fluoresence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GITC	Guanidine isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
HEV	High endothelial venule
HRP	Horseradish peroxidase
HSP	Heat shock protein
i.p.	Intraperitoneal(ly)
i.v.	Intravenous(ly)
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IκB	InhibitorκB
IL	Interleukin

ILN	Inguinal LN
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	Inducible nitric oxide synthase
KO	Knockout
LAL	Limulus Amebocyte Lysate
LB broth	Lubria-Bertani broth
LN	lymph node
LPS	Lipopolysaccharide
M.O.I	Multiplicity of infection
M-CSF	Macrophage-colony stimulating factor
MDC	Monocyte-derived dendritic cell
ME	Mercaptoethanol
MHC	Major histocompability complex
MIP-3α	Macrophage inflammatory protein-3 alpha
MLN	Mesenteric lymph nodes
mRNA	Message RNA
NADPH	Nicotinamide-adenine dinucleotide phosphate
NFκB	Nuclear factorκB
NK	Natural killer
NO	Nitric oxide
NP	Nucleoprotein
Nramp1	Natural resistance-associated macrophage protein 1
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
OmpA	Outer membrane protein A
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium iodide
PI3k	Phosphatidylinositol 3 kinase
PIP3	Phosphoinositide-3,4,5-trisphophate
PL	peritoneal lavage
PLN	Popliteal LN
PPs	Peyer's Patches
PS	Phosphatidylserine
ReA	Reactive arthritis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
S. typhimurium	*Salmonella enterica* serovar Typhimurium
SCV	*Salmonella*-containing vacuole
SE	Standard error
Slc11a1	Solute carrier family 11a member 1
SLO	Secondary lymphoid organ

SpA	Spondyloarthropathy
SPI-1	*Salmonella* Pathogenicity Island 1
SPI-2	*Salmonella* Pathogenicity Island 2
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TTSS	Type III secretion system
μg	Microgram
μl	Microlitre

Chapter 1: Introduction

1.1 Reactive Arthritis

1.1.1 Definition

Reactive Arthritis (ReA) is usually defined as an immune-mediated sterile synovitis triggered by a distant infection through the urogenital or gastrointestinal tract (Childs, 2004; Toivanen and Toivanen, 2004). Common infections triggering ReA include *Chlamydia trachomatis* and *Salmonella, Shigella, Yersinia*, and *Campylobacter* (Colmegna et al., 2004; Hughes and Keat, 1994). Another distinctive feature of ReA is its association with the MHC I molecule HLA-B27, for which ReA has been classified as one type of seronegative spondyloarthropathy (SpA).

SpA is a family of arthritides sharing certain clinical features, such as being rheumatoid factor negative in the sera, and having a strong association with HLA-B27 (Kim et al., 2005). Other members in this family include ankylosing spondylitis, psoriatic arthritis, arthritis related to inflammatory bowel diseases, juvenile spondyloarthritis as well as undifferentiated SpA. Ankylosing spondylitis is a progressive disease in which chronic inflammation can lead to extensive new bone formation throughout the spine, and in ankylosing spondylitis more than 90% of patients are HLA-B27 positive. Up to 70-90% of patients with ReA are HLA-B27 positive, and evidence has been presented that in HLA-B27 positive individuals the disease is more severe and the prognosis poorer (al-Khonizy and Reveille, 1998; Khan and Kellner, 1992; Leirisalo et al., 1982).

ReA is of interest to study because of its clear bacterial trigger and the strong association with HLA-B27, which makes it a suitable model to investigate the role of bacteria in immune-mediated disorders, and also to help clarify the pathogenesis of the whole group of SpA.

1.1.2 Epidemiology

ReA is seen all over the world, with a prevalence estimated to be 30-40 cases per 100 000 adults (Toivanen and Toivanen, 2004). Caucasians are affected more often than other ethnic groups, e.g., African American, by a 4:1 ratio (Childs, 2004). ReA is a common arthritis, affecting persons between the ages of 20 and 40 years. Three percent of all men with a sexually transmitted disease will develop ReA. Women are also at risk; however, they are nine times less likely to develop ReA after venereal infection and symptoms are often more subtle and less severe. Enteric infections precipitating ReA affect males and females equally (Childs, 2004; Kohnke, 2004).

1.1.3 Clinical Presentation

The most common first symptom is joint discomfort, which occurs usually one to three weeks following the infection. However, it is not unusual that the triggering infection such as diarrhea and urogenital infection has passed unnoticed for some patients. Therefore, it is important to precisely inquire about the past history of infection in order to distinguish it from other arthritis. ReA is a disease with systemic symptoms such as malaise, fever and fatigue with the arthritis as the leading symptom. The disease primarily affects the large joints of the lower

extremities, such as the knees, ankles, and feet, in an asymmetric, oligoarticular and additive nature. The severity of the arthritis varies from mild and transient swelling or discomfort to serious, severely invalidating conditions. Enthesitis and sacroilitis are also common presentations. In particular, the presence of enthesitis, inflammation of the ligaments and tendons at the sites of their insertion into the bone, is a helpful distinguishing characteristic. Low back pain and buttock pain are common and can signal spinal or sacroiliac involvement. Extraarticular presentations including skin and mucous symptoms and ocular lesions can be seen in some patients (Kohnke, 2004; Toivanen and Toivanen, 2004).

The prognosis of ReA is generally considered good. The disease is generally self-limiting, resolving in 3-12 months. Up to 15% of cases progress to chronic arthritis lasting longer than 12 months (Kohnke, 2004). Recurrences can occur in 15% of ReA cases, and approximately 10% of ReA patients may with time progress to ankylosing spondylitis (Kohnke, 2004). It has also been established that recurrences and chronic development are more common in HLA-B27 positive individuals (Leirisalo et al., 1982).

Non-steroidal anti-inflammatory drugs (NSAIDs) form the cornerstone of the treatment of ReA, and many patients will attain satisfactory control of symptoms with these agents alone. For chronic ReA, especially if progression to ankylosing spondylitis is suspected, disease-modifying anti-rheumatic drugs can be considered. Sulfasalazine and methotrexate are both beneficial in controlling joint pain and inflammation but need to be monitored. Recently, biological

agents blocking tumour necrosis factor (TNF)-α or its receptors are being increasingly used in the treatment of ReA (Reveille and Arnett, 2005). Additionally, as ReA is definitely caused by an infection, it is natural to consider antibiotic treatment. However, it has been concluded that a short conventional course of antibiotics is effective only if given early enough during the pathogenetic process of ReA. Later, after the process has started, antibiotics are of little if any value (Toivanen and Toivanen, 2004). Nevertheless, a recent follow-up of a clinical study has suggested that long-term antibiotic treatment may, especially in HLA-B27 positive individuals, reduce later morbidity (Yli-Kerttula et al., 2000).

1.1.4 Pathogenesis of ReA

1.1.4.1 Genetic factors

1.1.4.1.1 HLA-B27

HLA-B27 makes a strong genetic contribution to the development of SpA. The most striking association is observed for ankylosing spondylitis, 94% of patients express HLA-B27 compared with approximately 9% of the general population (Brown et al., 1996). The association of HLA-B27 with ReA is illustrated by the fact that the prevalence of disease in HLA-B27-positive individuals is five times greater than in the general population; in HLA-B27 relatives of patients with ReA, the prevalence is another 10 times greater (Feltkamp, 1995). Direct evidence comes from transgenic animals. HLA-B27 transgenic rats develop a multi-system disease with many features resembling the human HLA-B27-associated diseases (Hammer et al., 1990b). In addition, symptoms develop

upon transfer from a specific pathogen-free environment to conventional conditions (Khare et al., 1995). Moreover, in transgenic animals, susceptibility to disease is clearly related to the gene copy number and level of expression of HLA-B27 (Taurog et al., 1993), and requires the presence of HLA-B27+ bone marrow cells (Breban et al., 1993) and T cells (Breban et al., 1996), consistent with the features of immune mediated disease.

HLA-B27 is an MHC class I molecule, which comprises a glycosylated heavy chain (45Kda) non-covalently associated with β_2-microglobulin (β_2m) (12Kda). The class I heavy chain consists of three extracellular domains, α_1 (N-terminal), α_2 and α_3, the transmembrane region and a cytoplasmic tail. The α_1 and α_2 domains constitute a platform of 8 anti-parallel β strands supporting 2 anti-parallel α helices, which form a highly polymorphic peptide-binding cleft comprising of 6 pockets (A, B, C, D, E, F) (Bjorkman et al., 1987; Madden et al., 1991; Madden et al., 1992). The most distinctive structural feature of HLA-B27 is its B pocket, with a glutamic acid at its base and a cysteine residue at its mouth. This pocket is surrounded by a unique combination of residues that is found in all disease-associated HLA-B27 subtypes and confers the HLA-B27 B pocket with a strong specificity for an arginine side chain at the second position of bound peptides (Colbert et al., 1994). Moreover, another unique characteristic of the HLA-B27 molecule is the presence of an unpaired cysteine-67 residue within the extracellular α1 domain, which facilitates adherence of the two heavy chains to each other via disulfide binding to form a "homodimer" (Allen et al., 1999) (Bird et al., 2003; Bowness et al., 1999). These unique and

inherent "defects" suggest that HLA-B27 has abnormal cell biology, which may be of pathogenic significance.

Over 25 molecular subtypes of HLA-B27 have been described so far. The most common subtypes (HLA-B*2705, B*2702, B*2704, B*2707) are clearly associated with a risk for spondyloarthritis. Among them, B*2705 is the dominant subtype and is associated with spondylitis across broad ethnic and geographic boundaries. Two subtypes of HLA-B27, HLA-B*2706 (found in Southeast Asia) and B*2709 (found in Sardinia) appear not to be associated with spondylitis (Mody et al., 2003). This is possibly due to amino acid differences in the "C" or "F" pockets of the HLA antigen binding cleft which alter the composition of peptides presented by these HLA-B27 subtypes (Hulsmeyer et al., 2004; Montserrat et al., 2003).

Although HLA-B27 has remained a centre of extensive research, the exact mechanism underlying the effect of HLA-B27 on disease susceptibility still has not been determined. Moreover, why HLA-B27-associated diseases attack certain organ systems, such as the joint, the spine, the gut and the eye, and not others, is unknown. Several different hypotheses have been proposed.

Based on the major function of HLA-B27 as a class I antigen in presention of antigenic peptides to cytotoxic lymphocytes, the arthritogenic peptide model proposes that the disease is the product of a cytotoxic T cell response to a peptide found only in joint tissues that can specifically be bound and presented by all HLA-B27 subtypes through their shared structural motif. However, under

"normal" conditions the peptide is presented at levels too low for T-cell recognition either for clonal deletion or for initiating an immune response. Upon infection, bacteria may carry proteins with sequence motifs similar to the arthritogenic peptide. Infection would then sensitise T cells so that they could detect low levels of presentation of the arthritogenic peptide and initiate an anti-self response. From this point of view, SpA is an autoimmune response mediated by T cells against self antigens after a breakdown in self tolerance by the triggering bacterial infection (Benjamin and Parham, 1990). It was recently reported that CD4+ T cells may also be involved in class I-restricted immune recognition (Boyle et al., 2004), and consequently, SpA could involve an HLA-B27-restricted CD8+ or CD4+ T cell response to microbial or self-peptides.

The arthritogenic peptide hypothesis would require particularly stringent selection of unique peptide epitopes by HLA-B27. Peptide presentation by HLA-B27 is known to be influenced by subtype polymorphisms within the peptide binding groove. Although subtypes of HLA-B27 differ at other key positions within the peptide binding groove, their characteristic B pocket is conserved (Madden et al., 1991). Evidence for this hypothesis comes from the identification of HLA-B27-restricted peptides from *Chlamydia trachomatis* (Kuon et al., 2001), as well as from molecular mimicry between endogenous B27 peptides and environmental antigens (Dulphy et al., 1999; Fiorillo et al., 2000).

Recently, enthesitis has received more attention as the hallmark that distinguishes SpA from all other arthritides (Granfors et al., 2002). The cartilage proteoglycans versican and aggrecan and the link protein are being studied as

possible autoantigens in SpA (Khan, 2002). In a recent study, it was demonstrated that HLA-B27-restricted epitopes derived from human aggrecan are involved in the induction of typical inflammation of SpA (tenosynovitis) in BALB/c-B27 transgenic mice (Kuon et al., 2004). Overall, however, although the theory of "arthritogenic peptides" was received much attention, the arthritogenic capacity of candidate peptides has proved difficult to define.

There has been considerable interest in aberrant processing or folding of the heavy chain of HLA-B27. Under normal circumstances cell surface HLA-B27, consisting of a heavy chain bound to β_2m and peptide, is formed in the endoplasmic reticulum (Pamer and Cresswell, 1998). It has been recently described that, as a result of its unique B pocket architecture, newly synthesised HLA-B27 is slow to fold and associate with β_2m, and has a tendency to misfold (Ortmann et al., 1997). The accumulation of misfolded HLA-B27 protein may result in a proinflammatory "unfolded protein response" intracellularly in the endoplasmic reticulum, and contribute to the pathogenesis of HLA-B27-associated disease (Mear et al., 1999). In addition to the misfolding, heavy chains of HLA-B27 have been shown to refold to form disulfide-bonded homodimers in the absence of β_2m, which is dependent on the presence of an unpaired cysteine-67 residue (Allen et al., 1999). Furthermore, it has been shown that these homodimers of HLA-B27 heavy chains are expressed on the surface of human lymphoid cell lines (Bird et al., 2003) and in HLA-B27-positive patients with SpA (Kollnberger et al., 2002). HLA-B27 heavy chain homodimers can bind peptides and present them to T lymphocytes or natural killer cells, particularly when the cell's ordinary antigen-presenting

function is impaired (Kollnberger et al., 2004). Alternative recognition of different forms of HLA-B27 by leukocytes receptors could influence their immunomodulatory function and may imply a role in the development of SpA (Allen et al., 2001), but evidence is lacking to define the pathogenic significance of this interaction (Kim et al., 2005).

Recently, clear evidence that HLA-B27 itself is recognised by human CD4+ T cells isolated from SpA patients has been provided (Boyle et al., 2001). A variety of forms of the molecule, including conventional HLA-B27 heterodimers, free HLA-B27 heavy chain monomers, and unconventional homodiers are involved in this unusual recognition, which also is independent of MHC II and transporter associated with antigen processing (TAP). Supporting these findings, Roddis *et al* (Roddis et al., 2004) have recently demonstrated that CD4+ and CD8+ T cell responses induced in HLA-B27 restricted TCR transgenic mice (GRb) are both functional; whether HLA-B27-restricted CD4+ T cells also occur in patients and consequently play a pathogenic role requires further investigation. Adoptive transfer studies have shown that CD4+ T cells are more important than CD8+ T cells for induction of arthritis (Breban et al., 1996). Together, these observations suggest the role of unusual recognition of MHC I-restricted CD4+ T cells in the pathogenesis of SpA.

A recent hypothesis to explain the role of HLA-B27 in SpA proposes "auto-display" of HLA-B27 (Luthra-Guptasarma and Singh, 2004). In this hypothesis, β_2m-free, peptide-free heavy chains support a helix-coil transition from the α_2 domain to the α_3 domain, facilitating the rotation of backbone angles around

residues 167/168, thereby occupying the peptide binding cleft of the molecule. This auto-display of HLA-B27, occurring either within HLA-B27 molecules or between HLA-B27 molecules, might be the grounds for self-perpetuating inflammatory and immune stimulation, although experimental evidence proving the existence of such assemblies is lacking.

There is another theory involving β_2m deposition (Uchanska-Ziegler and Ziegler, 2003). This is based on the observation that disease associated HLA-B27 subsets exhibit a higher rate of β_2m dissociation from the surface HLA-B27 complex than non-associated subsets, which results in the deposition of β_2m in synovial fluid in the form of amyloid aggregates, causing immune response against such aggregates. However, with this particular hypothesis, HLA-B27 itself appears incidental rather than central to the disease mechanism. Moreover, transgenic animal studies point to the opposite results showing that β_2m deficiency (without expression of HLA-B27) is sufficient to cause spontaneous inflammatory arthritis (Kingsbury et al., 2000).

Intracellular persistence of arthritogenic microorganisms may contribute to the pathogenesis of ReA, suggesting the host/microbe interaction may be abnormal in susceptible individuals, leading to inefficient elimination of arthritogenic bacteria, fragments of them or both, after the initial infection. To support this notion, studies have shown that HLA-B27 expression decreases arthritogenic bacteria invasion into transfected L cells (Mear et al., 1999). In addition to invasion, HLA-B27 also remarkably impairs the elimination of *Salmonella enteritidis* within transfected human monocytic cells (Laitio et al., 1997). More

recently, HLA-B27-positive monocytes were shown to kill *Salmonella in vitro* less efficiently than controls, and they showed upregulated production of interleukin 10 (IL-10) and to a lesser extent, TNFα (Ekman et al., 2002; Inman and Payne, 2003), indicating that HLA-B27 associated modulation of cytokine response profiles may have importance in the pathogenesis of disease. From this point of view, the function of HLA-B27 may not be limited to antigen presentation. However, other studies have shown that arthritogenic bacteria fail to affect HLA-B27 peptide presentation (Ringrose et al., 2004), and invasion and persistence of *Salmonella* in fibroblasts are not affected whether the cells are isolated from HLA-B27 positive or negative individauls (Huppertz and Heesemann, 1997). The diverse results may reflect the properties of different cells and bacterial strains used in these studies, as both elements have a crucial effect on the outcome. Although the findings remain controversial, this theory takes account of the effects of triggering bacteria and genetic susceptibility into the pathogenesis of ReA, which is worthy of further investigation.

1.1.4.1.2 Other genes

Several studies have been reported on genes other than HLA-B27 in SpA (Zhang et al., 2004). An early study showed that HLA-B60 increased the risk of ankylosing spondylitis in HLA-B27-positive Caucasian patients (Robinson et al., 1989). More recently, it was further shown that HLA-B60 and B61 increased the susceptibility to ankylosing spondylitis in HLA-B27-negative patients (Wei et al., 2004). In addition, another study reported that HLA-DR1 is also associated with ankylosing spondylitis (Brown et al., 1998).

Large multifunctional proteases and transporters associated with antigen presentation (which act as chaperones for peptide transport) have also been studied in the context of ankylosing spondylitis, but with varying conclusions (Burney et al., 1994; Vargas-Alarcon et al., 2004). In addition, a number of other non-MHC candidate genes including IL-1 (Timms et al., 2004), TNF-α (Milicic et al., 2000), matrix metalloproteinase-3 (Jin et al., 2005) and transforming growth factor (TGF)-β (Howe et al., 2005) have been examined for susceptibility to SpA. The potential involvement of additional genes beyond HLA-B27 suggests a polygenic model of genetic susceptibility for SpA. It also offers an explanation for the fact that not all SpA patients display HLA-B27 expression.

1.1.4.2 Environmental factors

It is well established that ReA is a joint inflammation that follows infections caused by certain Gram-negative bacteria, as detailed above, although the triggering infection is less clear in other diseases of the SpA family. HLA-B27 transgenic rats in a germ-free environment do not develop inflammatory pathology in the gut or the joints, and induction of arthritis following reintroduction of commensal gut flora supports the notion that such organisms play an important role in the pathogenesis of HLA-B27 associated disease (Taurog et al., 1994).

1.1.4.2.1 Evidence of bacteria or their products in the joints

Evidence of *Chlamydia*, the commonest causative agent for ReA, in the joints has been provided using a number of different techniques. *Chlamydia* DNA, mRNA, rRNA, and intact *Chlamydia*-like cells (live but non-culturable) have been found in synovial tissues and peripheral blood (Bas et al., 1995; Gerard et al., 1998; Hammer et al., 1992; Kuipers et al., 1998; Schumacher et al., 1988; Taylor-Robinson et al., 1992). The presence of mRNA and rRNA (nucleic acids which have a half-life of minutes in tissues) of *Chlamydia* suggests the occurrence of transcription and hence active multiplication of the bacteria (Gerard et al., 1998). The fact that an inverse relationship between the presence of Chlamydial DNA in the joint and synovial *Chlamydia*-specific lymphocyte proliferation has been found (Sieper et al., 1999) suggests that an impaired T-cell response, possibly related to the expression of HLA-B27, might contribute to the persistence of bacteria.

Attempts to cultivate viable *Yersinia* or *Salmonella* from affected joints have failed. However, DNA of *Yersinia, Shigella,* or *Campylobacterer* has been identified in some studies (Granfors et al., 1990; Granfors et al., 1989; Hammer et al., 1990a) (Colmegna et al., 2004; Sibilia and Limbach, 2002). Antigens of these bacteria have been demonstrated in samples of synovial tissue or synovial fluid from patients (Granfors et al., 1989; Hammer et al., 1990a). Bacterial products including lipopolysaccharide (LPS) of *Yersinia, Salmonella, Shigella* (Granfors et al., 1990; Granfors et al., 1992; Granfors et al., 1989), *Chlamydia trachomatis* (Jendro et al., 2000), and the YadA protein (Hammer et al., 1990a) and heat shock protein (Granfors et al., 1998) of *Yersinia* have been identified in the joints from ReA patients. Moreover, *Yersinia* LPS and heat

shock protein in the synovial fluid and peripheral blood cells have been detected for up to 4 years in ReA patients (Granfors et al., 1998), *Salmonella* LPS has been found in synovial tissue 2 years after initial infection (Granfors et al., 1990), and the presence for several months of antibodies at high concentrations in serum samples from patients with ReA (Maki-Ikola et al., 1991) all indicate the persistence of bacteria or their products in the host after the onset of infection.

Microbes or their components may enter the synovial tissues via blood vessels either as whole organisms that may circulate in the blood (bacteraemia) or within the cells as a part of immune complexes (Colmegna et al., 2004; Granfors et al., 1990; Schumacher et al., 1988). It has been suggested that monocytes, including macrophages, may serve as a reservoir or as a transporter of bacteria to synovial tissue (Colmegna et al., 2004). This is supported by an important property of synovium, which is that approximately 50% of the cells that make up the lining layer are macrophages recruited from peripheral blood (Gaston, 1998). As tissue macrophages are differentiated from blood monocytes, bacteria or their components phagocytosed by the monocytes or macrophages at the site of infection (mucosa) or in the blood will inevitably find their way to the joints, with the dose being influenced by recruitment rates. Large joints and joints with some degree of inflammation due to mechanical stress would recruit greater numbers, and this factor might account for the preferential involvement of large lower limb joints in ReA. Some experimental evidence also supports this notion, since peripheral blood mononuclear cells that contain intracellular bacterial fragments are especially prone to bind to

synovial high endothelial venules and transmigrate through the endothelial cell monolayer (Kirveskari et al., 1998). In addition, monocytes that have engulfed ReA-triggering bacteria can induce the expression of P-selectin on cultured endothelial cells, and this molecule is important for homing to the synovium (Salmi and Jalkanen, 2001). However, despite these findings direct evidence that monocytes/macrophages serve as transporters of bacterial antigens to the joints is still lacking.

It is also likely that these bacteria survive at an extra-articular site, in particular in the mucosal membranes of the digestive system and/or the lymphocytic tissues, and are carried to the joint by monocytes, probably in recurrent fashion (Granfors et al., 1998; Kirveskari et al., 1999; Kirveskari et al., 1998). A recent study has depicted the dynamic picture of invasion, degradation and persistence of *Yersinia* and *Salmonella* in synovial fluid (Meyer-Bahlburg et al., 2001). In this study, Infection of synovial fluid with these bacteria was shown to start with the adhesion of the bacteria to synovial cell membrane and was followed by uptake of the morphologically intact bacteria into the cells. A few hours after infection, the bacteria showed very slow metabolic activity and started a process that finally led to the total disappearance of the bacterial cytosol. This may help to partially explain the clinical feature of frequent recurrences in some ReA patients.

1.1.4.2.2 Common features of triggering bacteria

The ReA-triggering bacteria share certain common biologic features: they are able to live intracellularly, they have LPS as an important component of the outer membrane, and they infect at a mucosal surface (Reveille and Arnett, 2005). It is still not clear how these different bacteria can lead to a similar clinical picture and have a similar association with HLA-B27. The identification of immunodominant antigens of these bacteria is of great importance to aid understanding of the pathogenesis. A common antigen shared by all bacteria has not yet been identified.

It has been suggested that the potential immunodominant antigen has to meet four requirements: first, it needs to be expressed in the synovial microenvironment; second, it has to be conserved across several bacterial species; third, cross-reactivity must exist between human and bacterial antigens; finally, it must enable bacteria to evade the immune response.

For years, an intensive search for the decisive arthritogenic antigen has been going on and so far not one has been found. Bacterial heat shock protein (hsp) 60 seems to be a major target of the T-cell response in ReA, and cross-reactivity against autologous hsp60 has been discussed as a cause of autoimmunity (Sieper et al., 2000). However, the T cells specific for hsp60 of *Chlamydia* fail to cross-react with hsp60 from enterobacteria (Deane et al., 1997). Alternative candidates have also been identified among immunodominant antigens in *Yersinia*-induced ReA, including a cationic 19-kDa urease B subunit and ribosomal protein L23. The fact that the urease subunit is highly conserved among bacteria supports the possibility that autoimmunity in

ReA may be mediated by antigenic mimicry between evolutionarily conserved epitopes (Mertz et al., 1994).

Rather than causing autoimmunity, bacterial LPS has been documented to play an important role in the pathogenesis of ReA because it contributes greatly to the virulence of the bacteria and acts to modulate the immune system. In patients with *Salmonella*-induced ReA, the persisting humoral immune response and intra-articular antibodies are directed primarily against LPS (Maki-Ikola et al., 1992). In addition, LPS in synovial tissue is a potent macrophage stimulator and can induce a range of inflammatory cytokines, largely via the nuclear factor-κB (NF-κB) pathway (Deng et al., 1999). LPS also induces the chemokine, monocyte chemotactic protein, enhances secretion of the polymorphonuclear leukocyte chemotactic and activating chemokine IL-8 from chondrocytes (Gaston et al., 1999), and decreases C5aR expression on monocytes (Gerard et al., 1998). All these changes lead to the recruitment of leukocytes into the synovium and to the persistence of activated macrophages within the synovium and the ensuing chronic inflammation.

Bacterial DNA which contains more nonmethylated CpG motifs than mammalian DNA, can intensely stimulate monocytes/macrophages, and thus may also contribute to inflammation. This idea is supported by a study showing that experimental intra-articular injection of bacterial DNA is sufficient to induce arthritis in mice (Deng et al., 1999).

1.1.4.3 Interaction of bacteria and host in ReA

1.1.4.3.1 Humoral immune response

Although bacteria-specific antibodies are of diagnostic value, the humoral immune response does not explain the immunopathogenesis and MHC-association of this disease. However, it may correlate with the severity and duration of the disease (Thomson et al., 1995). The production of IgM, IgG and IgA classes of antibody has been observed in *Salmonella*-triggered ReA (1991). The mainly humoral response is IgA (especially in enteric ReA), implying chronic stimulation of the gut mucosa (Kingsley and Panayi, 1992). The persistence of antibodies may indicate persistence of bacterial antigens in the arthritic patients, which also suggests an impaired cellular immunity (Kingsley and Panayi, 1992). Analysis of B cells isolated from lymphoid infiltrates from synovial tissue of ReA patients suggests that B cell differentiation occurs in an antigen-driven, T cell-dependent manner (Schroder et al., 1997). Nevertheless, the fact that antibody responses may be absent in ReA patients indicates that they play only a secondary role in the pathogenesis of synovitis (Kingsley and Panayi, 1992).

1.1.4.3.2 Cellular immune response

In the pathogenesis of ReA, T cells play a central role (Colmegna et al., 2004) and antigen-specific CD4+ and CD8+ T cells have been detected in synovial fluid of ReA patients (Beacock-Sharp et al., 1998). The function of HLA-B27 is to present peptides to cytotoxic T cells, and HLA-B27 restricted cytotoxic T cell clones against bacterial antigens have been demonstrated (Figure 1.1 a) (Hermann et al., 1993; Ugrinovic et al., 1997) (Appel et al., 2004a). In addition, multiple T lymphocyte expansions are found in both the blood and synovial fluid

of patients with ReA, and expansions are most commonly found in the synovial CD8+ compartment, where they appear to express both activation and memory markers (Allen et al., 1997). These results suggest that HLA-B27 restricted CD8+ T cells may be pathogenic.

However, studies with HLA-B27 transgenic mice have suggested that the role of HLA-B27 in joint disease may not simply be as a restriction element for CD8+ T cells, since HLA-B27 transgenic mice lacking β_2m have extremely low levels of CD8+ T cells but still develop inflammatory disease (Khare et al., 2001). Also induction of disease in these mice is independent of the *TAP-1* gene, which is required for the loading peptide onto MHC class I molecules (Khare et al., 2001). In addition, adoptive transfer studies have demonstrated that CD4+ T cells are more efficient in transferring inflammatory disease than CD8+ T cells when the separate T cell subsets are transferred to nude HLA-B27+ transgenic rats (Breban et al., 1996). These observations suggest that CD4+ T cells may be more important than CD8+ T cells in the pathogenesis of ReA.

Although early studies have found predominant HLA class II restricted CD4+ responses to the triggering organisms (Figure 1.1 d) (Hassell et al., 1993; Sieper et al., 1993), more recently it has been suggested that MHC II molecules are not required for disease development because MHC-II negative HLA-B27 transgenic mice still develop spontaneous disease (Khare et al., 1998). This raises the possibility that recognition of HLA-B27 by CD4+ T cells may be involved in disease pathogenesis. As discussed above, there is evidence that

such recognition occurs (Figure 1.1 b, c, e) (Boyle et al., 2004; Boyle et al., 2001; Roddis et al., 2004).

Various studies have focused on analysis of homodimers formed by free HLA-B27 heavy chain that is not associated with β_2m (Allen et al., 1999; Bird et al., 2003; Kollnberger et al., 2004). Expression of these homodimers has been found on the surface of human lymphoid cell lines (Allen et al., 1999; Bird et al., 2003) and in HLA-B27-positive patients with SpA (Kollnberger et al., 2002). It has been suggested that the formation of homodimers enables the binding of an altered repertoire of antigenic peptides, or might simply resemble the MHC II molecule leading to the recognition by CD4+ T cells (Bowness et al., 1999). In addition to their classical antigen-presenting role, different forms of HLA-B27, including either classic or free heavy chain monomer or homodimers, are recognised by members of the killer immunoglobulin receptor and leukocyte immunoglobulin-like receptor families (Allen et al., 2001). Therefore, as the ligands for paired Ig-like receptors, which are expressed on natural killer cells, the interaction of HLA-B27 with natural killer cells has been implicated (Figure 1.1 f); exactly how this interaction contributes to the disease pathogenesis remains to be shown.

Taken together, the accumulated data appear to show that CD4+ and CD8+ T cells may both play an important part in the pathogenesis of ReA, while role of HLA-B27 homodimer recognition by CD4+ T cells has drawn more recent attention.

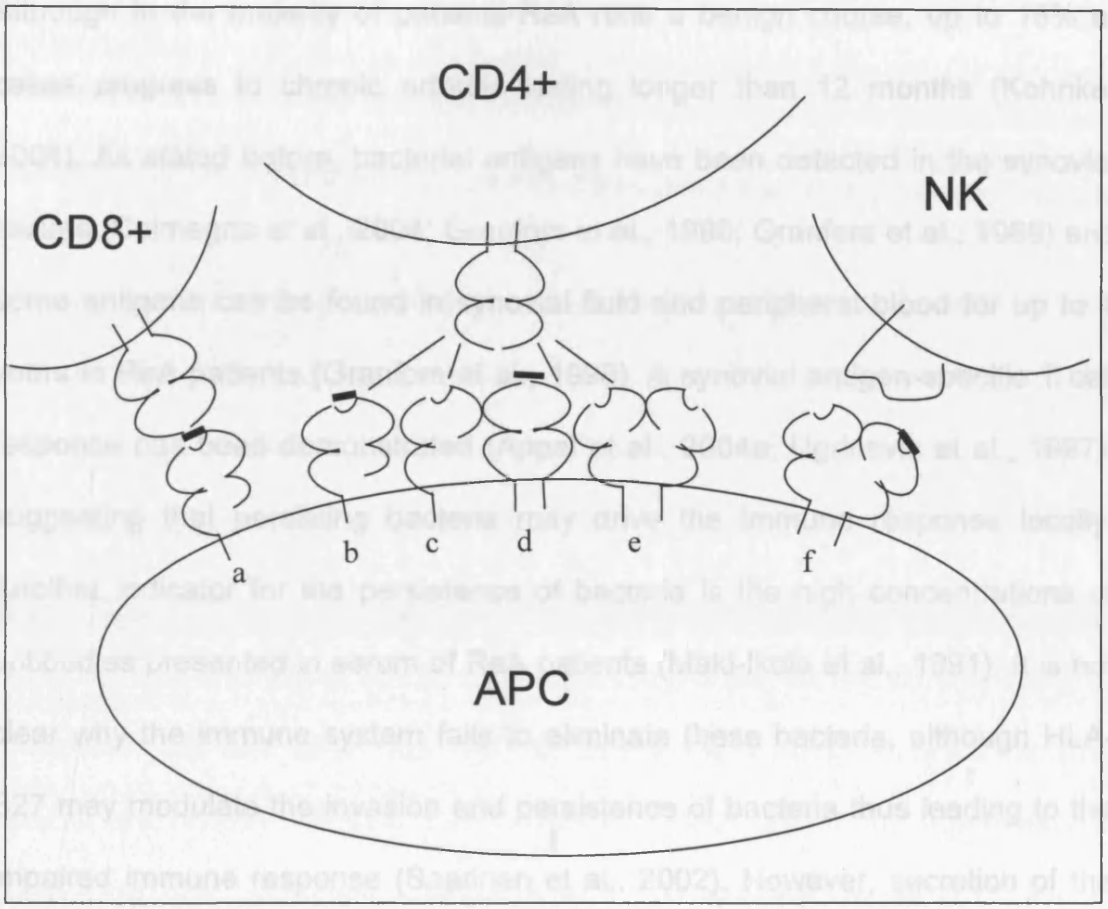


Figure 1.1 Schema for a number of possible recognitions related to HLA-B27 molecules in the pathogenesis of ReA. Adapted from Reveille *et al* (Reveille and Arnett, 2005). (a) HLA-B27/β2m and peptide complex recognised by CD8+ T cells. (b) HLA-B27/β2m and peptide complex recognised by CD4+ T cells. (c) Free HLA-B27 heavy chain alone reacting with CD4+ T cells. (d) HLA class II (DR, DQ, DP) presenting HLA-B27 restricted peptide to CD4+ T cells. (e) HLA-B27 homodimers reacting with CD4+ T cells. (f) HLA-B27 homodimers recognised by receptors on NK cells.

1.1.4.3.3 Cytokines

Although in the majority of patients ReA runs a benign course, up to 15% of cases progress to chronic arthritis lasting longer than 12 months (Kohnke, 2004). As stated before, bacterial antigens have been detected in the synovial tissues (Colmegna et al., 2004; Granfors et al., 1990; Granfors et al., 1989) and some antigens can be found in synovial fluid and peripheral blood for up to 4 years in ReA patients (Granfors et al., 1998). A synovial antigen-specific T cell response has been demonstrated (Appel et al., 2004a; Ugrinovic et al., 1997), suggesting that persisting bacteria may drive the immune response locally. Another indicator for the persistence of bacteria is the high concentrations of antibodies presented in serum of ReA patients (Maki-Ikola et al., 1991). It is not clear why the immune system fails to eliminate these bacteria, although HLA-B27 may modulate the invasion and persistence of bacteria thus leading to the impaired immune response (Saarinen et al., 2002). However, secretion of the appropriate cytokines is known to be crucial for an effective immune response to eliminate bacterial infection (Yin et al., 1997). Among cytokines active in host defense against intracellular bacteria, Th1 and Th2 cytokines are of particular importance. Thus, the Th1 and Th2 balance has been suggested to determine the outcome of infection with the ReA-associated bacteria (Burmester et al., 1995). TNF-α and IFN-γ are potent antibacterial Th1 cytokines and involved in promoting cell-mediated immunity, which is required for an effective cellular immune responses against intracellular bacteria, whereas IL-4, IL-5, IL-9 and Il-13 are Th2 cytokines and more involved in the generation of humoral immunity and allergic responses. In addition, production of Th2 cytokines also has an

inhibitory effect on the function of Th1 cytokines (Hitchon and El-Gabalawy, 2002; Paul and Seder, 1994).

The issue of which cytokine secretion pattern predominates the milieu of synovial fluid of ReA patients is controversial. An early study showed that a Th1 cytokine pattern was predominant in synovial fluid of patients with *Yersina* induced ReA (Schlaak et al., 1992), which has been confirmed by another recent study (Butrimiene et al., 2004). High production of TNF-α and IFN-γ detected in chronic ReA supports the possible use of anti-TNF-α treatment, which has shown a promising effect in the clinic, especially for cases of chronic ReA (Reveille and Arnett, 2005). On the contrary, impaired Th1 (TNFα) cytokine production has also been observed to correlate with longer disease duration (Braun and Sieper, 1999). Concomitantly, there are some other studies suggesting that cytokines associated with Th2 subsets (such as IL-4) are prominent in the synovium of patients with ReA (Simon et al., 1994; Yin et al., 1997), which may delay elimination of bacteria, thus leading to persistence of the pathogen. In addition, since the disease resolves in the majority of patients with ReA (although often taking 12 months to do so) (Kohnke, 2004), another explanation for the significance of a detectable Th2 cytokine profile in the development of disease is the appearance of anti-inflammatory Th2 cytokines, which may function to control joint inflammation (Gaston, 1998). This notion is supported by a study of animal models of arthritis showing that IL-4 can down-regulate joint inflammation (Joosten et al., 1997). The differences in synovial tissue cytokine profiles demonstrated in the above studies are difficult to reconcile, and may relate to patient selection, sampling of the synovial tissue, or

in vitro processing techniques. The underlying mechanism that determines the differentiation of T helper lymphocytes, thus resulting in a skewing of the cytokine secretion profile, is unclear. The local inflammatory milieu, the amount of IL-12 during T cell priming, differences among antigen-presenting cells (APCs) and antigen dose have been discussed as likely factors (Moser and Murphy, 2000; Paul and Seder, 1994; Sher and Coffman, 1992).

Another subset of CD4+ T lymphocytes producing IL-10 and TGF-β has been proposed to have a regulatory role, and an elevated level of IL-10 and TGF-β secretion has been detected in the synovial membrane of ReA patients (Appel et al., 2004b). Whether this is a general phenomenon, and how it affects the Th1 and Th2 balance, remains to be determined.

1.2 Macrophages

Macrophages are bone marrow derived cells that enter the blood as monocytes, which, after approximately a day in the blood stream, enter and reside in several different tissues and organs where they differentiate into resident macrophages. Macrophages are actively phagocytic and endocytic cells, playing a key role in innate immunity because they can recognise, ingest, and destroy many pathogens without the aid of an adaptive immune response. They are the first line of host defense to encounter pathogens. Macrophages recognise pathogens by means of cell-surface receptors including the macrophage mannose receptor, scavenger receptors, glucan receptors and Toll-like receptors. (Aderem and Underhill, 1999; Takeuchi et al., 1999; Underhill et al., 1999). Phagocytosis by macrophages can be dramatically

enhanced through binding of pathogens with Fc receptors and complement receptors, a process called opsonisation. After phagocytosis, macrophages produce a variety of toxic products that help kill the engulfed microorganism. The most important of these are reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are generated by lysosomal nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases and other enzymes in a process known as the respiratory burst. The second important effect of the interaction between pathogens and macrophages is activation of macrophages to release cytokines and other mediators that establish a state of inflammation in the tissue and recruit large numbers of macrophages from the blood to the site of local inflammation. Due to their phagocytic capacity as well as their ability to upregulate MHC and co-stimulatory molecules upon activation, macrophages also have an important role in antigen presentation. Finally, macrophages are an important effector of cell-mediated responses following exposure to inflammatory cytokines such as IFN-γ produced by T cells, which eventually facilitate the elimination of the invading pathogens.

1.3 Dendritic cells

Dendritic cells (DC) are a sparsely distributed, migratory group of bone-marrow derived leukocytes that are specialised for the uptake, transport, processing and presentation of antigens to T cells (Shortman and Liu, 2002). Owing to their distribution throughout most tissues of the body and being widely accepted as the most potent APCs in the immune system, DC are positioned at the centre of innate and adaptive immunity and have a prominent role in the initiation of the

primary immune response compared to other APCs such as macrophages (Steinman and Nussenzweig, 2002).

Recent evidence suggests that in both mice and man there exist at least two major subsets of DC, namely conventional DC and plasmacytoid DC (PDC) (Cavanagh and Von Andrian, 2002; Klechevsky et al., 2005). Conventional DC are the classical T cell priming subset and may ultimately shape the type of T cell response following microbial invasion (Klechevsky et al., 2005). The function of PDC is less clear. These cells produce large amounts of IFNα in response to viral infection and therefore may play an important role in defence against pathogens (Colonna et al., 2002).

Several subpopulations have been described among conventional DC in mice. DC are broadly defined by expression of CD11c and MHC class II, but can be further divided into subpopulations based on differential expression of the CD8α homodimer and CD4. Three subpopulations have been defined which are present in the spleen and lymph nodes: CD8α+CD4-, CD8α-CD4+ and CD8α-CD4- (Moser and Murphy, 2000; Shortman and Liu, 2002). Some functional differences between these subpopulations have been reported. For example, CD8α+ DC have been shown to be superior in inducing a Th1-biased cytokine response, endocytosing apoptotic cells and cross-presentation; whereas, CD8α- DC tend to induce a Th2-biased response (Shortman and Liu, 2002). In addition to these three subsets additional DC subsets are present in lymph nodes and other tissues (Shortman and Liu, 2002). This includes Langerhans

cells, a distinctive DC subtype found in skin, which are characterised by expression of langerin (Shortman and Liu, 2002).

DC exist in distinct stages of maturation depending on signals received from their environment. Immature DC reside predominantly in peripheral tissues in the absence of inflammatory stimuli or microbial products. They have a high capacity for antigen uptake and processing, but a relatively poor capacity to stimulate naive T cells (Banchereau and Steinman, 1998) (Banchereau et al., 2000). Immature DC express low levels of MHC class I and MHC class II molecules and costimulatory molecules such as CD80, CD86, CD40 and the adhesion molecule CD54 (ICAM-1) that are all important for interaction with T cells. In addition, immature DC also express chemokine receptors that bind to chemokine ligands that are considered to be pro-inflammatory, which are important for positioning and recruitment of different DC subsets into different peripheral tissues (Cravens and Lipsky, 2002). Immature DC monitor their environment for signs of danger and can interact directly with microbial stimuli through pattern recognition receptors such as Toll-like receptors, and can take up antigens via macropinocytosis or, in the case of apoptotic cells, by phagocytosis (Seya et al., 2001).

Toll-like receptors (TLRs) are innate receptors that sense microbial products and trigger DC maturation and cytokine production, thus effectively bridging innate and adaptive immunity (Napolitani et al., 2005). These receptors signal in response to conserved microbial components such as LPS by TLR4, flagellin by TLR5, unmethylated CpG oligonucleotides by TLR9, and double-stranded RNA

by TLR3, and therefore are thought to be critical components of the immune response (Napolitani et al., 2005). DC express a broad repertoire of TLRs through which they can recognise a wide range of microbial components. In addition, cytokines such as IL-1β, TNF-α or engagement of CD40, are also able to trigger DC to mature (Banchereau et al., 2000; Banchereau and Steinman, 1998).

The process of maturation leads to the downregulation of the antigen capture capacity, increased surface expression of costimulatory molecules, enhanced cytokine secretion and altered chemokine responsiveness. The latter involves the downregulation of chemokine receptors that bind to pro-inflammatory chemokines and an up-regulation of the chemokine receptor, CCR7 (Ohl et al., 2004), that binds to the chemokine CCL19 and CCL21 constitutively expressed in distinct sites of SLO (Miyasaka and Tanaka, 2004). This property allows DC migration from inflamed tissues to SLO and hence optimal positioning to encounter antigen-specific T cells.

For experimental use, murine DC can be obtained by two ways. BMDC can be generated by cultivation of bone marrow cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) for 6-8 days (Inaba et al., 1992; Lutz et al., 1999). The cells from this culture represent a heterogenous population of DC, consisting of immature DC, defined as CD11c+ MHC II ^{low}, and mature DC, which are CD11c+ MHC II ^{high} (Cheminay et al., 2002; Lutz et al., 1999). Alternatively, splenic DC can be purified from spleens by digestion with collagenase, after which CD11c+ cells are enriched by positive selection

using magnetic beads. This method also applies DC purification from lymph nodes.

1.4 *Salmonella*

1.4.1 Serovar

The genus *Salmonella* is extremely heterogeneous, comprising almost 2000 serotypes, of which only a few are major human pathogens. The serotype differences are based on differences in surface antigens such as LPS and flagella (Monack et al., 2001). Pathogenic *Salmonella* species are an important cause of infectious diseases throughout the world. *Salmonella* serovars are responsible for human diseases that range from self-limiting gastroenteritis to systemic infections. The strict human pathogen, *Salmonella enterica* serovar Typhi (*S. typhi*), causes typhoid fever, a systemic disease, which results in 16 million illnesses and 600,000 deaths worldwide each year (Jones, 2005). Transmission of this disease within the human population is generally a result of poor sanitation of water and food supplies in developing countries. Whereas *Salmonella enterica* serovars Enteriditis and Typhimurium cause the majority of human gastroenteritis infections, up to 1.3 billion cases of acute gastroenteritis are caused by non-typhoid salmonellosis, leading to 3 million deaths annually (Pang et al., 1995). These broad host-range *Salmonella* serovars are prevalent within warm-blooded animal populations that make up the human food supply and bacterial transmission generally results from consumption of raw or undercooked food products (Darwin and Miller, 1999). Therefore, these high numbers of infections emphasise the importance of this intracellular pathogen.

While mortality due to *Salmonella* infections is mainly a health problem in developing countries, morbidity due to acute *Salmonella* infections also has important socio-economic impact in developed countries (Hansen-Wester and Hensel, 2001).

1.4.2 Significance of Salmonella study

In addition to its clinical importance, *Salmonella* is an interesting model organism for the study of host-pathogen interaction on the molecular level. *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is frequently used for this purpose, since this organism has a broad host range and causes disease in a variety of animals. It causes a self-limited gastroenteritis in humans and a systemic typhoid-like disease in mice, thus serving as an experimental model for typhoid fever. Moreover, this model also illustrates that mice are more susceptible to *S. typhimurium* than humans, which is probably controlled by genes that include SLc11a (Fortier et al., 2005). Furthermore, *Salmonella* spp. are interesting candidates for use as live recombinant vaccines and may be useful for the mucosal delivery of recombinant vaccine antigens (Hopkins et al., 2000).

S. typhimurium infection of mice and *S. typhi* infection of humans are characterised by inflammation at the site of bacterial entry and in deeper infected tissues (Jones and Falkow, 1996). *Salmonella* invasion results in characteristic tissue changes, including infiltration of neutrophils and monocytes/macrophages, which contribute to the erosion of the intestinal

mucosa (Darwin and Miller, 1999). After *Salmonella* transverses the epithelial barrier, it preferentially infects phagocytes within the lamina propria and subsequently enters the lymphatics and bloodstream and spreads to the liver and the spleen (Vazquez-Torres et al., 1999).

1.4.3 Salmonella and ReA

Salmonella is one of the most common ReA inducing agents, as 33% enteric ReA is triggered by *Salmonella* infection (Colmegna et al., 2004). According to previous *Salmonella* outbreak reports, 1-15% of patients with *Salmonella* gastroenteritis develop ReA as a postinfection complication (Inman et al., 1988; Locht et al., 1993). Of the 2000 different *Salmonella* serotypes, 22 have been reported to trigger ReA, although about 60% of ReA cases are triggered by *S. typhimurium* (Maki-Ikola O, 1991).

1.4.4 Major virulence mechanisms of Salmonella

Salmonella has evolved sophisticated virulence mechanisms to infect and colonise their hosts and these functions are exerted through bacterial type III secretion systems (TTSS). TTSS are specialised organelles that translocate bacterial virulence proteins (effectors) from the bacterial cytoplasm directly into the host-cell cytoplasm (Kimbrough and Miller, 2002). These translocated effectors alter such basic host-cell functions as signal transduction, cytoskeletal architecture, membrane trafficking, and cytokine gene expression (Waterman and Holden, 2003). Two distinct virulence-associated TTSS have been described, and they perform different functions during *Salmonella*-host

interaction. The *Salmonella* Pathogenicity Island 1 (SPI-1) locus is a 40-kb gene cluster at centisome 63 of the *Salmonella* chromosome (Galan and Curtiss, 1989). It delivers at least 13 effector proteins through the host cell plasma membrane, most of which are involved in actin cytoskeleton rearrangement, leading to membrane ruffling, thus resulting in *Salmonella* invasion of intestinal epithelial cells or facilitating uptake of organism into a *Salmonella*-containing vacuole (SCV) (Waterman and Holden, 2003). In addition, SPI-1 effectors induce IL-8 and pathogen-elicited epithelial chemoattractant secretion in intestinal epithelial cells, resulting in transmigration of neutrophils (Lee et al., 2000). The SPI-1 translocon protein SipB binds to and activates caspase-1, leading to the induction of apoptosis in macrophages (Hersh et al., 1999).

Following invasion, *Salmonella* infection of the intestinal mucosa leads to phagocytosis by macrophages; survival and proliferation inside macrophages that eventually renders the development of systemic disease (Fields et al., 1986). The intracellular phase of *Salmonella* infection is characterised by processing of the SCV along a unique path of vesicular trafficking, which requires expression of the genes in SPI-2, as well as loci controlled by the two component regulatory system PhoPQ (Holden, 2002). The SPI-2 TTSS, which encompasses a 25 kb cluster of genes at centisome 30.7 of the *Salmonella* chromosome, encodes a second TTSS that delivers virulence effector proteins produced by organisms in the SCV across the vacuolar membrane and into the cytoplasm of the host cell (Waterman and Holden, 2003). SPI-2 has been shown to be required for inhibition of various aspects of endocytic trafficking, including fusion between lysosomes and SCVs (Uchiya et al., 1999), an

avoidance of NADPH oxidase-dependent killing by macrophages (Gallois et al., 2001; Vazquez-Torres et al., 2000b), delayed apoptosis-like cell death (van der Velden et al., 2000), control of SCV membrane dynamics (Beuzon et al., 2000; Ruiz-Albert et al., 2002), the assembly of a meshwork of F-actin around the SCV (Meresse et al., 2001), cholesterol accumulation around the SCV (Catron et al., 2002) and interference with the localisation of inducible nitric oxide synthesas to the SCV (Chakravortty et al., 2002).

Analysis by electron microscopy revealed that the TTSS encoded by SPI-1 is a membrane-bound assembly with a typical needle-like structure, termed the "needle complex" (Kubori et al., 1998). Although there is no direct experimental evidence, it can be assumed that the SPI-2-encoded TTSS shows structural and functional similarity to the SPI-1 system. The "needle complex" is a long hollow structure about 120 nm long and is composed of two clearly identifiable domains: a needlelike portion projecting outward from the surface of the bacterial cell and a cylindrical base that anchors the structure to the inner and outer membranes (Figure 1.2). Biochemical analysis of purified needle complex revealed that they are composed of at least three proteins: InvG (a member of the secretin family) and two lipoproteins, PrgH and PrgK (Galan and Collmer, 1999) (Figure 1.2 D). It is thought that the TTSS "needle complex" serves as a hollow conduit through which the type III secreted proteins are delivered to the host cell cytoplasm (Galan and Collmer, 1999). More recently, by using time-lapse microscopy, delivery of the *Salmonella* type III effector proteins such as SopE into animal cells in real time has been imaged (Schlumberger et al., 2005). This mechanism of *S. typhimurium* has been used to deliver

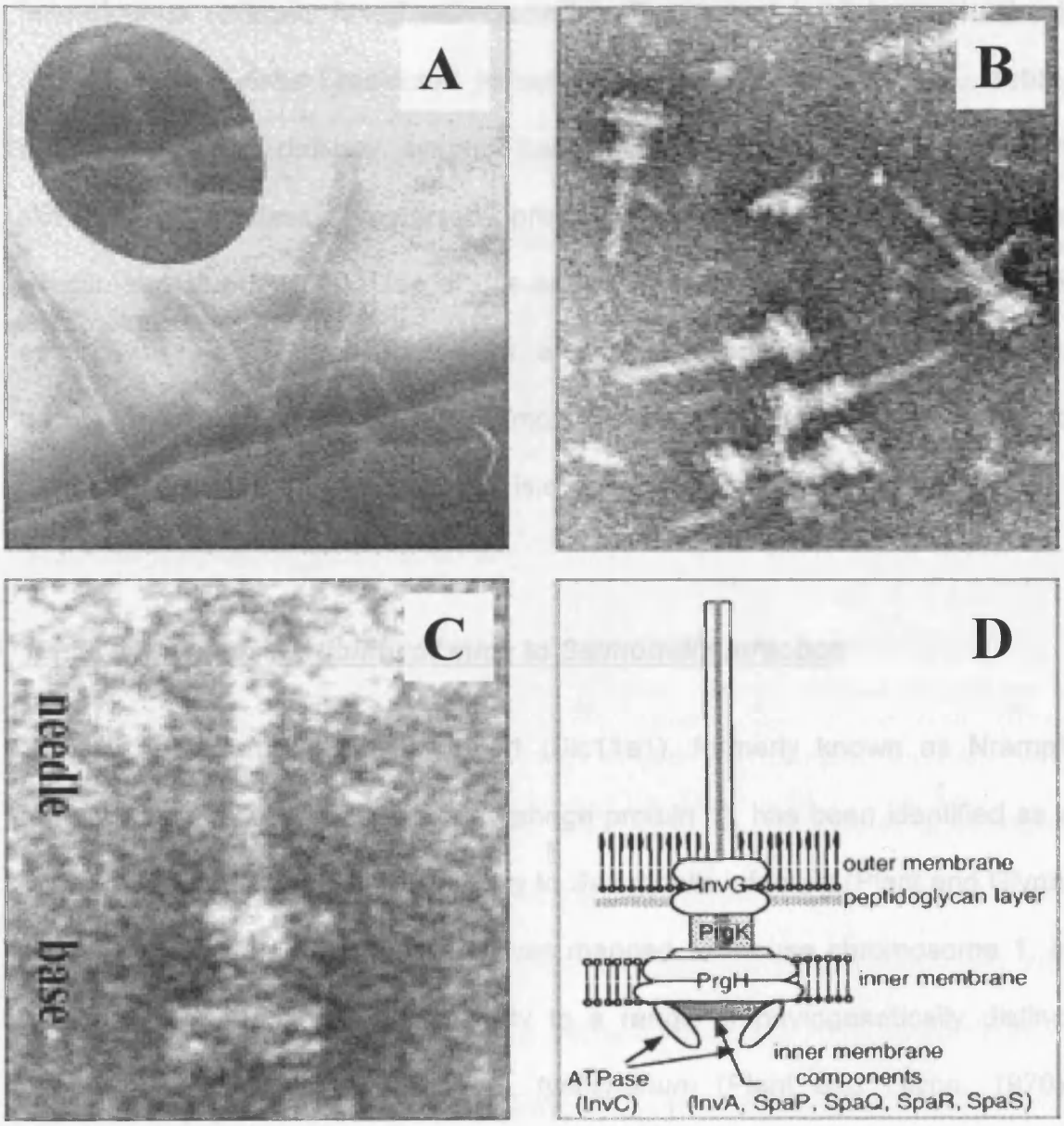


Figure 1.2 Electron micrographs of needle complexes from osmotically shocked *S. typhimurium* on the bacterial envelope (A), purified (B), enlarged (C), and schematic representation and its major components (D). These graphs are adapted from Galan *et al* (Galan and Collmer, 1999) and Kimbrough *et al* (Kimbrough and Miller, 2002).

heterologous epitopes fused with bacterial effector proteins into the host-cell cytosol to elicit class I-restricted immune response (Russmann et al., 1998), and this antigen delivery system has been shown to result in efficient stimulation of class I-restricted protective antiviral immune responses (Russmann et al., 1998). Use of this antigen delivery system will expand the efficient use of *S. typhimurium* as a carrier of heterologous antigens to vaccinate against infections and/or tumour diseases in which a class I-restricted Cytotoxic T cell lines (CTL) response is crucial for protection.

1.4.5 Genetic susceptibility of mice to Salmonella infection

Solute carrier family 11a member 1 (Slc11a1), formerly known as Nramp1 (natural resistance-associated macrophage protein 1), has been identified as a major regulator of mouse susceptibility to *Salmonella* infection (Plant and Glynn, 1974; White et al., 2004). Slc11a1 was mapped to mouse chromosome 1. In mice, this gene confers susceptibility to a range of phylogenetically distinct intracellular pathogens including *S. typhimurium* (Plant and Glynn, 1976), *leishmania donovani* (Bradley et al., 1979), and *Mycobacterium bovis* BCG (Gros et al., 1981). A naturally occurring Gly-Asp mutation at amino acid 169 of Slc11a1 in mice leads to unrestricted bacterial replication in the liver and spleen, resulting in a lethal infection of *Salmonella* (Vidal et al., 1995b). In human, Slc11a1 is associated with infectious and autoimmune diseases (Blackwell et al., 2003).

Identification of the mouse Slc11a1 found that the Slc11a family was an ancient family, encoding proteins with high homology to membrane-bound transporter proteins, which is highly conserved from yeast to humans (Cellier et al., 1996). The mammalian Slc11a family is divided into two classes, Slc11a1 and Slc11a2. Slc11a1 is exclusively expressed in the late endosomes/lysosomes and phagosomes of phagocytic cells such as monocytes/macrophages and granulocytes (Govoni et al., 1997; Vidal et al., 1995a). Slc11a2 ubiquitously localizes to early endosomes in macrophages (Gruenheid et al., 1999) and other cell types throughout the body.

Although Slc11a1 was identified before Slc11a2, the biochemical function of Slc11a2 has been determined, while that of Slc11a1 remains unclear. Using *Xenopus* oocytes, it was shown that Slc11a2 protein is a pH-dependent divalent cation transporter, capable of transporting divalent cations including Fe^{2+} and Mn^{2+} (Gunshin et al., 1997). The same study claimed that Slc11a1 can also transport Fe^{2+} and Mn^{2+}, but with less efficiency than Slc11a2. More recently, another study has suggested that Slc11a1 is a more efficient transporter of Mn^{2+} than Fe^{2+} (Fortier et al., 2005). In conjunction with their localizations, a potential model for iron homeostasis in macrophages was proposed. Slc11a2 in early endosomes delivers extracellularly acquired divalent cations (Fe^{2+}) into the cytosol; and Slc11a1 in late endosomes/lysosomes delivers divalent cations from the cytosol to this acidic compartment. Subsequently, the Fenton and or Haber-Weiss reaction can use ferrous iron to generate toxic hydroxyl (OH•) radicals (Zwilling et al., 1999). This model provides one mechanism by which Slc11a1 could directly contribute to antimicrobial activity in macrophages.

However, some other studies proposed that Slc11a1 also delivers divalent cations into the cytosol (Fortier et al., 2005; Gomes and Appelberg, 1998; Jabado et al., 2000). Conversely, Goswami and colleagues (Goswami et al., 2001) demonstrated that Slc11a1 can flux divalent cations in either direction depending upon the pH on either side of the membrane. Hence the direction of transport of divalent cations by Slc11a1 remains controversial.

Slc11a1 mediated divalent metal ion transport at the vesicular compartments has many pleiotropic effects on macrophage activation, including induction of inducible nitric oxide synthesas (iNOS), nitric oxide (NO) release, L-arginine flux, oxidative burst and regulation of cytokines such as IL-1β, TNF-α (Blackwell et al., 2003). These effects appear to be crucial for Slc11a1 mediated resistance *in vivo*. In addition, Zaharik et al have demonstrated that the presence of Slc11a1 up-regulates the expression of SPI-2 virulence genes (*ssrA*, *sseA* and *sseJ*), which are critical for intra-macrophage survival of bacterium in Slc11a1-transfected cell lines and murine systemic *Salmonella* infection (Zaharik et al., 2002). Hence, this delineates an active interplay between SPI-2 and the innate genetic host defence mechanism during infection.

Although numerous studies have attempted to elucidate the true biochemical function of Slc11a1 in macrophages, a clear and definitive answer has not been achieved. In addition, further studies required to determine the underlying molecular basis of disease susceptibility mediated by Slc11a1 in human diseases.

1.4.6 T cell responses in Salmonella infection

It has been clearly shown that T cells play a central role in the control of *S. typhimurium* infection, with B cells also contributing (Mittrucker and Kaufmann, 2000). Although γδ T cells represent a large fraction of T cells in the intestinal epithelium, and depletion of γδ T cells results in increased susceptibility to oral *Salmonella* infection, mice with depletion of αβ T cells show a more profound defect (Mixter et al., 1994), which suggests that αβ T cells are more important for protective immunity against *Salmonella* infection. Depletion of CD8+ T cells from donor cells reduces the capacity to passively transfer protection against virulent *S. typhimurium,* indicating a specific role for CD8+ T cells (Mastroeni et al., 1992; Nauciel, 1990). By differentiation into cytolytic T cells, which then produce cytokines such as IFN-γ and TNF-α and release granulysin, a protein with the direct antibacterial activity (Stenger et al., 1998), CD8+ T cells are able to lyse infected cells. This leads to release of bacteria from their protective environment, as well as contributing directly in bacterial killing.

Various data indicate that CD4+ T cells are more important than CD8+ T cells in anti-*Salmonella* response, as transfer of CD4+ T cells from vaccinated mice into naive recipients resulted in higher levels of protection than transfer of CD8+ T cells (Mastroeni et al., 1992; Nauciel, 1990; Pie et al., 1997). In addition, mice deficient in CD4+ T cells (MHC II-deficient mice) failed to clear infection with an attenuated strain of *S. typhimurium* and developed chronic disease (Hess et al., 1996). In general, CD4+ T cells function by differentiating into Th1 and Th2 cells, characterised by secretion of distinct cytokines. Studies have demonstrated that infection of mice with *Salmonella* induces a Th1 response,

indicated by the production of large amounts of IFN-γ (Ramarathinam et al., 1991; Thatte et al., 1993). The importance of IFN-γ in the control of *Salmonella* was demonstrated in mouse mutants deficient in the IFN-γ receptor, which were highly susceptible to attenuated *Salmonella* (Hess et al., 1996). *Salmonella*-specific Th1 cells are most likely the major source of IFN-γ during the later stages of infection, and especially during the secondary response (Mittrucker and Kaufmann, 2000). Th1 cells can also produce TNF-α (Mastroeni et al., 1992; Tite et al., 1991), with additional sources of TNF-α being macrophages. TNF-α also plays a critical role during *Salmonella* infection, since neutralisation of TNF-α *in vivo* can prevent the host from mounting a protective response, and the animals succumb to the infection (Everest et al., 1998; Mastroeni et al., 1993). Th2 cytokines that have been analysed in *Salmonella* infection are IL-4 and IL-10 (Pie et al., 1996; Pie et al., 1997). These are not involved in protection, but rather impair control of *Salmonella* infection (Mittrucker and Kaufmann, 2000).

1.5 Chemokines and chemokine receptors

Chemokines are small (around 10 kDa) secreted chemotactic cytokines that regulate the migration of leukocytes under steady-state and inflammatory conditions (Sozzani, 2005). More than 40 chemokines have been identified so far (Rot and von Andrian, 2004). Based on their amino acid composition, 4 chemokine families have been described (Table 1.1), C, CC, CXC and CX3C, according to the motif displayed by the first two of four cysteine residues at the N terminus of the molecule. CXC and CC are the two major families, with the two cysteine residues separated by a single amino acid or in adjacent positions.

Table 1.1 Chemokines and their receptors and their classification

Chemokine	Original names	Receptor	Class
CC chemokine			
CCL1	I-309, TCA-3	CCR8	D
CCL2	MCP-1, MCAF, JE	CCR2	I
CCL3	MIP-1α	CCR1, CCR5	I
CCL4	MIP-1β	CCR5	I
CCL5	RANTES	CCR1, CCR3, CCR5	I
CCL6 (mouse)	MRP-1	CCR1	I
CCL7	MCP-3, MARC	CCR1, CCR3, CCR5	I
CCL8	MCP-2	CCR3	I
CCL9 (mouse)	MRP-2, MIP-1γ	CCR1, CCR2, CCR3	I
CCL10 (mouse)	CCF18	unknown	unknown
CCL11	Eotaxin	CCR3	I
CCL12 (mouse)	MCP-5	CCR2	I
CCL13	MCP-4	CCR2, CCR3	I
CCL14	HCC-1, Ckβ-1	CCR1	I
CCL15	HCC-2, Lkn-1, MIP-5	CCR1, CCR3	I
CCL16	HCC-4, LEC, Mtn-1, LCC-1	CCR1	I
CCL17	TARC	CCR4	D
CCL18	DC-CK1, PARC	unknown	H
CCL19	ELC, MIP-3β	CCR7	H
CCL20	MIP-3α, LARC	CCR6	D
CCL21	6Ckine, SLC, CKβ-9, TCA-4	CCR7	H
CCL22	MDC, STCP-1	CCR4	D
CCL23	MPIF-1, CKb-8	CCR1	I
CCL24	MPIF-2, Eotaxin-2	CCR3	I
CCL25	TECK	CCR9	D
CCL26	Eotaxin-3	CCR3	I
CCL27	CTACK, ILC, ALP, Eskine	CCR10	I
CCL28	MEC	CCR3, CCR10	I
CXC chemokine			
CXCL1	GROα, MGSA, MIP-2, KC	CXCR2	I
CXCL2	GROβ, MIP-2α	CXCR3	I
CXCL3	GROγ, MIP-2β	CXCR4	I
CXCL4	PF-4	unknown	unknown
CXCL5	ENA-78, LIX	CXCR2	I
CXCL6	GCP-2, CKα-3	CXCR1, CXCR2	I
CXCL7	NAP-2	CXCR	I
CXCL8	IL-8	CXCR1, CXCR2	I
CXCL9	MIG	CXCR3	D
CXCL10	IP-10, CRG-2	CXCR3	D
CXCL11	I-TAC	CXCR3	D
CXCL12	SDF-1	CXCR4	H
CXCL13	BLC, BCA-1	CXCR5	H
CXCL14	BRAK, bolekine	unknown	H
CXCL15 (mouse)	Lungkine	unknown	unknown
CXCL16		CXCR6	D
XC chemokine			
XCL1	Lymphotactin, SCM-1, ATAC	XCR1	I
XCL2		XCR2	I
CX3C chmokine			
CX3CL1	Fractalkine, Neurotactin	CX3CR1	I

Chemokines can be classified as inflammatory (I), homeostatic (H) or dual function (D) according to their expression pattern and function (Kim, 2004; Moser et al., 2004; Paixao et al., 2005) (McColl, 2002).

C and CX3C chemokines are minor families, which include two with a single cysteine residue and one with three amino acids separating the two cysteine residues (Moser et al., 2004).

Chemokines play a critical role in the directed movement of leukocytes from the bloodstream to tissues as well as localisation of cells within the tissues. However, emerging data have shown that various members of the chemokines exert a range of biological effects beyond chemotaxis, including angiogenesis, hematopoiesis, morphogenesis and autoimmunity (Cravens and Lipsky, 2002; Moser et al., 2004).

According to their expression patterns and various functions, chemokines can also be divided into three groups termed "inflammatory", "homeostatic" and "dual-function" chemokines. Inflammatory chemokines, for example CXCL8 and CCL5, expressed in inflamed tissue, control the recruitment of effector leukocytes in infection, inflammation, tissue damage and tumours. Many of them have broad target-cell selectivity and act on cells of the innate and adaptive immune system. Homeostatic chemokines, for example CCL19, CCL21, CXCL13, expressed constitutively in SLO, navigate leukocytes during hematopoiesis in the bone marrow and thymus, during initiation of adaptive immune responses in the SLO, and in immune surveillance of peripheral tissues. In contrast, dual-function chemokines have been defined more recently due to several chemokines with overlapping functions that cannot be assigned to either of the above two groups. They participate in immune defence functions

and also target precursor and resting mature leukocytes at sites of leukocyte development and immune surveillance (Moser et al., 2004).

Chemokines exert their various biological functions by binding to specific receptors, which are a group of G-protein coupled receptors (GPCR). GPCR are proteins with seven transmembrane spanning domains and are coupled to G proteins. The heterotrimeric G proteins are composed of three subunits, α, β and γ (Rot and von Andrian, 2004). After binding of the chemokines to the amino terminus of the receptors, the activated G-proteins dissociate into α- and $\beta\gamma$-subunits. The $\beta\gamma$-subunits activate the phosphotidylinositol-3 kinases (PI3K), which leads to generation of phosphoinositide-3,4,5-trisphosphate (PIP3). PI3K and its product PIP3 translocate and colocalise with the small GTPase Rac. PIP3 activates Rac through specific guanine nucleotide exchange factors, which subsequently enhances actin polymerisation and activates the focal adhesion apparatus, inducing formation of lamellipodia and filopodia. This reorganises the cytoskeleton of the cells, promoting the polarisation toward the concentration gradient of chemokines, resulting in cell motility and chemotaxis (Maghazachi, 2000; Rot and von Andrian, 2004).

In addition to activation of signalling pathways, GPCRs also regulate the process of desensitisation, which can occur upon prolonged and repeated exposure to chemokines (Kohout et al., 2004). Again, the heterotrimeric G proteins coupled to GPCRs dissociate into α and $\beta\gamma$ subunits. The latter recruit G protein coupled receptor kinases, which phosphorylate the carboxy terminus of the receptors. Phosphorylated receptors bind β-arrestins, which recruit non-

receptor tyrosine kinases c-Src, Lyn, Fyn or Syk. These in turn may phosphorylate dynamin, which binds clathrin-coated endocytic pits, facilitating the internalisation of the receptors. This pathway results in the termination of the signals (Maghazachi, 2000). Such agonist-induced phosphorylation of chemokine receptors followed by internalisation of the receptors is called "homologous desensitisation" (Ali et al., 1999). Chemokine receptors may also be subjected to "heterologous desensitisation" when a receptor loses its responsiveness following phosphorylation by second messager-activated kinases, i.e. protein kinase A or protein kinase C, which have been activated by unrelated receptors or signalling processes (Ali et al., 1999). For example, activation of formyl peptide receptor, the receptor for the bacterial chemotactic peptide fMLF, effectively desensitises a number of chemoattractant receptors, including the receptors for the chemokine CXCL8/IL-8 (Ali et al., 1999). Both homologous and heterologous desensitisation results in diminished responsiveness of chemokine receptors to further stimulation by their cognate ligands (Le et al., 2001).

"Homologous desensitisation" of a number of chemokine receptors, induced using their specific agonists, has been used as a tool in various studies. For example, CCR2 on the murine monocytoid WEHI78/24 cell line has been shown to be desensitised by 40 minutes preincubation with 500 nM CCL2/MCP-1 (Palframan et al., 2001). Similarly, CCR7 on murine splenic effector T cells could be desensitised by incubation for 60 minutes with up to 5 μg/ml of CCL19-Ig fusion protein (Unsoeld et al., 2004). Furthermore, as co-ligands for CCR7,

the ability of CCL19 and CCL21 to desensitise CCR7 on transfected cells has been compared (Kohout et al., 2004).

1.6 The role of macrophages during *S. typhimurium* infection

Macrophages play important roles in different stages of *S. typhimurium* infection. A genetic factor for the potential of macrophages to kill *S. typhimurium* is the expression of functional SLc11a 1. Deletion of functional SLc11a 1 protein in certain mouse strains dramatically reduces the efficacy with which macrophages kill *S. typhimurium*, resulting in a high susceptibility to infection (Fortier et al., 2005; Govoni et al., 1999; Vidal et al., 1995b). During the initial stages of infection, macrophages phagocytose bacteria, produce reactive oxygen and nitrogen intermediates and secrete pro-inflammatory cytokines such IL-6, IL-12 and TNF-α to control bacterial replication (Eckmann and Kagnoff, 2001; Norimatsu et al., 2003; Yrlid et al., 2000). However, this is not simple one-way process directed by the macrophages alone. By means of the TTSS, bacterial proteins are injected into the host cells, allowing *S. typhimurium* to interfere with the signalling machinery (Gallois et al., 2001; Schwan and Kopecko, 1997), promoting intracellular survival of the pathogen and causing apoptosis of infected macrophages (Chen et al., 1996a; Hersh et al., 1999; Schwan et al., 2000). Macrophages also contribute to anti-*Salmonella* immunity as APCs. Studies have shown that macrophages are able to process and present peptides on MHC-I and MHC-II molecules to CD8+ and CD4+ T cells. In the following sections, a few aspects of the interaction between macrophages and *Salmonella* infection are highlighted and introduced.

1.6.1 Invasion and survival

S. typhimurium infect the host through the intestinal tract and cross the epithelial barrier at the level of the ileum or the colon preferentially by invading M cells (Jepson and Clark, 2001; Jones et al., 1994). This cell type, which functions to sample luminal antigens, overlies the lymphoid follicles (Neutra et al., 1996a). The bacteria subvert the normal function of M cells and are taken up by inducing membrane ruffling in these cells, which requires the invasion-associated genes of SPI-1 (Collazo and Galan, 1997; Lee et al., 1992; Lostroh and Lee, 2001). After passing epithelium, the bacteria reach the subepithelial dome of the Peyer's Patch (Jones et al., 1994) and encounter an extensive network of resident macrophages and DC (Jones and Falkow, 1996; Neutra et al., 1996b). However, an alternative M cell-independent invasion mechanism has been demonstrated, whereby *S. typhimurium* is transported from the gastrointestinal tract to the bloodstream by CD18-expressing phagocytes, possibly consisting of DC and/or tissue macrophages (Vazquez-Torres et al., 1999). After being engulfed by phagocytes, *S. typhimurium* are thought to principally reside and multiply inside macrophages (Santos and Baumler, 2004; Weinstein et al., 1984). Mutants which fail to survive within macrophages are avirulent (Fields et al., 1986). In addition, Finlay et al have demonstrated by confocal microscopy that *S. typhimurium* colocalised *in vivo* with macrophages and resided intracellularly inside macrophages in the liver at later stages of disease (Richter-Dahlfors et al., 1997).

1.6.2 Cross talk: NADPH system and type III secretion system

Since *S. typhimurium* survive and replicate inside macrophages, remaining confined inside vacuoles in the infected cells (Oh et al., 1996), macrophages play a particular role in the pathogenesis of *Salmonella* infection by providing such protected sites for intracellular replication and a means of extraintestinal dissemination (Vazquez-Torres et al., 2000a). Soon after internalisation, F-actin is depolymerised from the phagosome, and the newly denuded vacuole membrane becomes accessible to early endosomes (Aderem and Underhill, 1999). Through a series of fusion and fission events, the vacuolar membrane and its contents mature, fusing with the late endosome and ultimately lysosomes, to form a phagolysosome. Following phagocytosis, two biochemical pathways contribute to the ability of macrophages to eliminate intracellular pathogens (Aderem and Underhill, 1999).

The NADPH phagocyte oxidase is among the most effective antimicrobial weapons. This enzymatic complex catalyses the univalent reduction of molecular oxygen to superoxide, a radical with modest antibacterial activity that serves as a precursor to more toxic derivatives including hydrogen peroxide and hydroxyl radical, which are collectively known as ROS. ROS have recently been shown to play a role in intracellular signalling (Ehrt et al., 2001). Like superoxide, NO is also synthesised in infected macrophages by a multicomponent enzyme, and is the precursor of a range of toxic radicals and ions, collectively known as RNS. RNS exert direct microbicidal and microbistatic effects (MacMicking et al., 1997). NO also plays a role in signalling (Coleman, 2001). In innate immunity, NO is required for IL-12 activation of natural killer

(NK) cells (Diefenbach et al., 1999) and is implicated in several other immunoregulatory roles and iron metabolism in macrophages (Bogdan, 2001; Bogdan et al., 2000). Fang and colleagues (Vazquez-Torres et al., 2000a) have demonstrated that the ROS and RNS both contribute to the anti-*Salmonella* activity of macrophages. ROS dependent killing is confined to the first few hours after phagocytosis, whereas RNS contribute to both early and late phases of anti-bacterial activity. RNS initially synergize with ROS to kill *S. typhimurium* and subsequently exert prolonged oxidase-independent bacteriostatic activity.

Rather than being destroyed by phagocytes after being engulfed, *Salmonella* has evolved several mechanisms to survive in the harsh milieu of macrophages mainly by utilising SPI-2 TTSS. After uptake, *Salmonella* resides in the SCV within macrophages. First, the SCV is acidified within an hour of uptake (Garvis et al., 2001; Rathman et al., 1997), which is required for *Salmonella* replication, probably because a low pH is necessary for secretion of SPI-2 proteins (Beuzon et al., 1999; Nikolaus et al., 2001). Second, *Salmonella* actively maintain and modify the vacuole and turn it into a "spacious phagosome" characteristic of owning a tightly opposed membrane. SifA (Brumell et al., 2001) and SseJ (Ruiz-Albert et al., 2002), two SPI-2 TTSS effector proteins are required in this process. Third, maturation of the SCV is disturbed and wild-type *S. typhimurium* rapidly becomes inaccessible to incoming endocytic materials and remains segregated from lysosomes and/or late endosomes (Garvis et al., 2001; Rathman et al., 1997). It has been reported that another SPI-2 effector, SpiC is involved in this process (Uchiya et al., 1999). Finally, *S. typhimurium* has several enzymes defending it from oxidative stress, which include the

periplasmic superoxide dismutases: sod CI and sod CII (De Groote et al., 1997; Fang et al., 1999; Farrant et al., 1997), and glucose 6-phosphate dehydrogenase (Lundberg et al., 1999). The latter is required for NADPH synthesis, which allows *Salmonella* to maintain its redox state and repair oxidative damage; another TTSS effector protein, SspJ, may also be involved (van der Straaten et al., 2001). No SPI-2 proteins have been found that are specifically to defend RNS, although the SPI-2 TTSS plays a role in limiting the bacteriostatic effects mediated by iNOS (Chakravortty et al., 2002).

1.6.3 Cytotoxicity and apoptosis

Chen and colleagues have shown that *Salmonella* is cytotoxic for cultured macrophages *in vitro* (Chen et al., 1996a). The cytotoxicity is initially manifested by an inhibition of membrane ruffling and macropinocytosis in infected macrophages, and is followed by cell death. The cytotoxic effects are strictly dependent upon the expression of the invasion-associated SPI-1 TTSS, because bacteria with mutations in invJ, spaO, sipB, sipC and sipD fail to induce cytotoxicity. Growth of *S. typhimurium* under conditions that do not allow optimal expression of TTSS also abolishes the cytotoxicity (Chen et al., 1996a). As mentioned above, SPI-1 is required to control bacterial invasion at early stages of infection (Baumler, 1997). It has been shown that the invasion process triggers a signal transduction pathway within macrophages that induces apoptosis (programmed cell death) (Monack et al., 1996). In addition, Zychlinsky et al have reported that *Salmonella* invasin SipB induces macrophages apoptosis by binding to caspase-1 (Chen et al., 1996b; Hersh et

al., 1999), suggesting a potential mechanism. Caspases are a family of host cell cysteine proteases, intimately involved in the initiation and execution of "classical apoptosis". They are synthesised as precursors that are converted to the active enzyme form by proteolytic processing (Hersh et al., 1999). Caspases participate in both the initiation of apoptosis and the process of cell disassembly in response to apoptotic stimuli. The exact mechanisms of caspase-mediated cell death are still not known yet.

Recent publications indicate that *S. typhimurium* grown to stationary phase do not induce the early caspase-1 killing but, instead, can kill macrophages 18-24 hour postinfection (Libby et al., 2000; Lindgren et al., 1996; van der Velden et al., 2000). The later stage of macrophage death exhibits some characteristics of apoptosis (Libby et al., 2000). Notably, SPI-2 proteins are required to induce this late macrophage death (van der Velden et al., 2000), indicating that *Salmonella* uses distinct TTSS to induce cell death at different stages of infection.

The ability of *Salmonella* to promote apoptosis may be important for the initiation of infection, bacterial survival and dissemination. The rapid SPI-1 and caspase-1-dependent cell killing in the gut-associated lymphoid tissue results in the processing and release of the proinflammatory cytokines IL-1β and IL-18 (Hersh et al., 1999; Mastroeni et al., 1999), which likely are responsible for the rapid inflammation and recruitment of additional phagocytic cells, along with their trafficking thus leading to dissemination. In contrast, once dissemination and replication have occurred, macrophages are killed by SPI-2-dependent

delayed killing in systemic tissue, and bacteria are released from dying macrophages, which may lead to further spread of bacteria and initiation of acquired immune response.

However, some recent studies have raised the question of whether *Salmonella*-induced cell death occurs by necrosis or apoptosis (Brennan and Cookson, 2000; Watson et al., 2000). Conventionally, apoptosis has a characteristic morphological appearance: cell shrinkage, membrane blebbing, intense perinuclear condensation, cytoplasmic vacuolisation, maintenance of organelle structure, and eventually the formation of apoptotic bodies. In addition, lack of inflammation is another hallmark of death by apoptosis. In contrast, necrotic cells exhibit a loss of membrane integrity and an enlargement of the nucleus and cellular organelles like mitochondria. Widespread necrosis is pro-inflammatory *in vivo* (Majno and Joris, 1995). Although apoptosis and necrosis are considered as distinct forms of cell death, there is increasing evidence that the morphological and biochemical characteristics of classical apoptosis and necrosis represent only the extreme ends of a wide range of possible deaths (Nicotera et al., 1999) and they may share common molecular pathways. Moreover, the two processes have been found to be functionally linked (Nicotera et al., 1999; Tsujimoto et al., 1997). Thus, it is sometimes difficult to distinguish between apoptosis and necrosis (Hirsch et al., 1997; Leist et al., 1997). Based on these difficulties, Falkow et al just refer to *Salmonella*-induced macrophage cytotoxicity as host cell death rather than applying any precise term to the mechanism (Monack et al., 2001).

1.6.4 Antigen Presentation

The antigen presentation capacity of macrophages is less potent than DC. It has been shown that macrophages and DC both are able to phagocytise and process bacteria for presentation of bacterial peptides on both MHC-I and MHC-II (Pfeifer et al., 1993; Pfeifer et al., 1992; Svensson et al., 1997b; Svensson and Wick, 1999). Co-incubation of BMDC or BMMac with *S. typhimurium* has been shown to result in upregulation of number of cell surface antigens including CD80, CD86, MHC-I and MHC-II, and this response does not require internalisation of the bacteria or that the bacteria are viable (Yrlid et al., 2000). More recently, the study of Villarreal-Ramos and colleagues showed that DC infected with *S. typhimurium* up-regulated cell surface expression of MHC-I, MHC-II, CD40, CD80 and CD86, while in contrast, infected macrophages did not exhibit detectable changes in expression of cell surface molecules, except for a increase in CD40 (Norimatsu et al., 2003). Due to insufficient surface expression of MHC and co-stimulatory molecules, macrophages are less efficient in triggering naïve T cells and initiating primary immune responses than DC. BMMac and peritoneal elicited macrophages can process and present bacterial antigens on MHC-II, but the cells need to be stimulated with IFN-γ to induce sufficient levels of MHC-II expression; in contrast, BMDC do not require IFN-γ pre-treatment to present antigen on MHC-II (Yrlid et al., 2000; Yrlid et al., 2001b).

The antigen processing and presentation pathways used by macrophages and DC may not be identical. For instance, for presentation of bacterial antigens on MHC-I, DC use a cytosolic pathway, which requires newly synthesised MHC-I,

the proteasome and TAP. Conversely, at early stages after infection, BMMac and peritoneal-elicited macrophages use a non-cytosolic pathway, which does not require the protease and TAP; at a later time, the cytosolic pathway may contribute significantly (Yrlid et al., 2000). The cytosolic pathway is the best characterised MHC class I antigen presenting pathway, while the mechanisms involved in the non-cytosolic pathway are not fully understood. Peptide regurgitation, a process of loading *Salmonella*-derived peptides to post-Golgi, but not newly synthesised, MHC-I molecules might be involved (Yrlid et al., 2000).

Besides direct antigen presentation, indirect antigen presentation also contributes to the pathogenesis of *Salmonella* infection. This is because *S. typhimurium* induces large amounts of macrophage cell death (as discussed earlier). Wick and Yrlid have demonstrated that bystander DC but not macrophages present antigens from bacterial-induced apoptotic macrophages, despite the fact that both cell types internalise apoptotic macrophages (Yrlid and Wick, 2000). This difference may be due to enhanced degradative capacity of macrophages compared to DC such that antigenic epitopes are destroyed in the macrophages containing apoptotic material (Linehan and Holden, 2003). In addition, the *Salmonella* virulence system pho-P regulates gene products that could decrease the processing and presentation of *S. typhimurium* antigens by activated macrophages (Wick et al., 1995).

1.7 The roles of dendritic cells during *Salmonella* infection

DC play an important role during *Salmonella* infection, especially with respect to linking innate and acquired immune responses. In the following section, several aspects of DC function during *Salmonella* infection will be discussed.

1.7.1 Entry and dissemination

DC play a key role in initiating immune responses to bacterial infections including *S. typhimurium*. The natural route of *Salmonella* infection is oral, most commonly in contaminated food. Thus, the intestinal tract is the first barrier the bacteria face. Subsequently, *S. typhimurium* invades the intestinal mucosa mainly through specialized epithelial cells, called M cells, that are located in the epithelium overlaying Peyer's patches (Jepson and Clark, 2001; Neutra et al., 1996a). Penetration of M cells by bacteria requires the expression of invasion proteins encoded by SPI-1 (Collazo and Galan, 1997; Lee et al., 1992; Lostroh and Lee, 2001). However, *S. typhimurium* that are deficient in invasion genes encoded by SPI-1 are still able to reach the spleen after oral administration (Galan and Curtiss, 1989), which suggests an M cell-independent pathway. Non-invasive *Salmonella* are unable to invade CD18-deficient mice, implicating CD18-expressing phagocytes including DC and macrophages in this alternative route (Vazquez-Torres et al., 1999). Hopkine et al demonstrated by confocal microscopy analysis that attenuated *Salmonella* were internalised and survived in Peyer's patches DC after oral inoculation (Hopkins et al., 2000). A more recent study provided further evidence that DC are able to open the tight junctions between epithelial cells, send their dendrites into the lumen of the gut

and directly sample bacteria (Rescigno et al., 2001), which confirms a novel bacterial uptake mechanism in the mucosal epithelium. More recently, Niess *et al* show that this uptake is regulated by CX_3CL1, a chemokine produced by intestinal epithelial cells (Niess et al., 2005), which provides an efficient way for DC to acquire antigen for presentation and might enhance the immune response against *Salmonella* infection.

After invasion, *Salmonella* uses SPI-2 TTSS to facilitate their intracellular survival and replication and induce late-stage apoptosis of infected host cells (Waterman and Holden, 2003). Although the macrophage is the predominant cell type to be used by *Salmonella* to achieve this function, some studies have demonstrated that *Salmonella* also efficiently enter and survive within cultured CD11c+ DC (Marriott et al., 1999a) and murine Peyer's patches DC (Hopkins et al., 2000). In addition, this survival does not depend on virulence factors required for intracellular survival in macrophages (Hopkins et al., 2000). Moreover, a significant level of apoptosis of DC has been observed after *Salmonella* infection, which occurs in a dose-dependent manner (Yrlid et al., 2001b). In concert with their unique localisation in the subepithelial dome of Peyers' patches (Iwasaki and Kelsall, 2000) and ability to be mobilised upon infection, DC may also serve as an important host cell for dissemination of this pathogen from mucosal sites (Marriott et al., 1999a; Vazquez-Torres et al., 1999). This is similar to a dissemination role documented in *L. monocytogenes* infection (Pron et al., 2001).

1.7.2 Maturation upon S. typhimurium infection

After encountering *S. typhimurium*, immature DC can be activated and converted into a mature state, indicated by several aspects. (1) Up-regulation of surface expression of MHC and costimulatory molecules, such as MHC I, MHC II, CD86, CD40 and CD54 (Niedergang et al., 2000; Svensson et al., 2000). Neither bacterial viability nor internalisation is required for this process to occur (Rescigno et al., 2001). (2) Secretion of cytokines upon bacterial interaction. Recent studies have shown that splenic DC produce TNF-α and IL-12 after co-culture with *S. typhimurium* (Marriott et al., 1999a; Yrlid and Wick, 2002). Again, neither bacterial internalisation nor viability is required for cytokine production. Yrlid *et al* concluded that *Salmonella* infection was also able to induce BMDC to release cytokines, but unlike for splenic DC, phagocytosis was required for BMDC cytokine production (Svensson et al., 2000; Yrlid et al., 2000). (3) Altered chemokine responsiveness. *Salmonella* infection is able to stimulate intestinal epithelial cell lines to constitutively express CCL20/MIP-3α mRNA (Izadpanah et al., 2001). CCL20/MIP-3α is a member of the CC chemokine subfamily, expressed by the follicle-associated epithelium overlaying the Peyer's patches (Iwasaki and Kelsall, 2000), which selectively chemoattract immature DCs that express the cognate receptor CCR6 (Liao et al., 1999; Power et al., 1997) to the subepithelial region of mucosal surfaces (Dieu et al., 1998; Iwasaki and Kelsall, 2000; Sallusto et al., 1998). In addition, CCR6-expressing DC are found in tissues close to CCL20-expressing epithelial cells or keratinocytes (Caux et al., 2000; Iwasaki and Kelsall, 2000). In CCR6 knockout mice, subepithelial myeloid DC are absent in the dome of Peyer's patches, and mucosal immune responses are impaired (Cook et al., 2000). More recent research has identified flagellin as

the major molecular trigger of enteropathogenic salmonellosis (Zeng et al., 2003). Furthermore, it has been shown that *Salmonella* flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of DC (Sierro et al., 2001). Therefore, during *Salmonella* infection, CCL20-dependent DC trafficking may serve as an important mechanism to recruit immature DC to the site of infection and subsequently initiate adaptive immune response in the gut. In contrast, it has been demonstrated that upon encounter with *S. typhimurium*, BMDC upregulate chemokine receptors CCR7 mRNA and are subsequently attracted towards the chemokines CCL19 and CCL21 (Cheminay et al., 2002). Upregulated CCR7 expression enables DC to traffic out of the tissues, and migrate to the blood and SLO (MartIn-Fontecha et al., 2003; Ohl et al., 2004), where they encounter antigen specific T cells.

1.7.3 Antigen presentation

As the most important consequence of maturation and migration, DC are competent to present antigen to T cells in SLO. DC present *Salmonella* derived antigens on MHC I and MHC II to CD8+ and CD4+ T cells respectively (Yrlid et al., 2001a; Yrlid et al., 2000; Yrlid et al., 2001b). This has been demonstrated for bone marrow derived DC (Svensson et al., 1997b; Svensson and Wick, 1999) as well as freshly isolated splenic DC (Yrlid et al., 2001a). Furthermore, Yrlid et al have demonstrated that splenic DC isolated from *Salmonella*-infected mice present bacterial antigens on MHC-I and MHC-II to antigen specific CD8+ and CD4+ T cells *ex vivo* (Yrlid and Wick, 2002). In addition, cytochalasin D (CCD) inhibition experiments have revealed that presentation by both pathways

requires phagocytosis of the bacteria (Yrlid and Wick, 2002). This is the case for MHC-I and MHC-II presentation pathways of BMDC as well (Yrlid and Wick, 2002).

Although *Salmonella* infection can induce apoptosis and necrosis of BMMac and BMDC (Yrlid et al., 2001b; Yrlid and Wick, 2000), cell death in infected cells does not necessarily mean avoiding the induction of specific immunity, because in addition to direct presentation of *Salmonella* antigens by DC and macrophages, bystander DC also take up apoptotic materials from macrophages that have undergone apoptosis, and present processed *Salmonella*-derived OVA antigens by MHC-I and MHC-II pathways respectively (Yrlid and Wick, 2000). It has been demonstrated that this mechanism requires virulent wild-type *S. typhimurium* in a logarithmic growth state to be used in the infection. Otherwise, apoptosis failed to be induced in macrophages. In addition, viability of bystander DC is another prerequisite as fixed cells failed to present antigen. Of note, to ensure the observed presentation was not due to direct presentation by infected macrophages, different MHC background macrophages were used and similar results were generated. As discussed in section 1.6.4, this pathway is unique to DC, since bystander macrophages were not able to do so. The existence of indirect antigen presentation suggests that apoptosis induced by microbes can lead to an immune response rather than to a "silent" nonimmunogenic death.

1.8 Chemokines and DC trafficking

It is generally believed that DC precursors in the peripheral blood migrate into peripheral tissues and differentiate to become immature DC. The fact that mature DC are constantly migrating from peripheral tissues to draining lymph nodes suggests that there must be a constitutive process of recruitment of DC precursors in order to maintain a consistent density of DC in peripheral tissues. It has been suggested that CCR2 and its ligands are involved in the initial movement of myeloid DC precursors into peripheral tissues (Vanbervliet et al., 2002). Plasmacytoid precursor DC cells have also been shown to express CXCR4 and migrate in response to CXCL12, indicating a role of this chemokine receptor in DC precursor entry into tissues (Zou et al., 2001). Different tissues produce a different profile of basal chemokine expression, which in combination with the differential chemokine receptor expression on DC precursors is likely to affect the rate of DC entry and the type of DC precursors entering specific tissues. This area requires more research for a better understanding regarding which molecules are involved in precursor DC recruitment.

After entering the tissues, precursor DC differentiate into immature DC, which make up the majority of DC in peripheral tissues. Immature DC express an array of chemokine receptors (Table 1.2). The receptors that bind to inflammatory chemokines include CCR1, CCR2, CCR3, CCR5, CXCR1, CXCR2 and CXCR3. The receptors that bind to homeostatic chemokines are CXCR4 and CXCR5 with the receptors CCR4, CCR6 and CCR8 that bind to dual function chemokines CCL17, CCL20 and CCL1 respectively.

Table 1.2 Chemokine receptor expression on immature and mature MDC and PDC and their ligand specificity

Receptor	MDC		PDC		Ligand/s
	Immature	Mature	Immature	Mature	
CCR1	+	-	-	ND	CCL3/5/7
CCR2	+	-	+	-	CCL2/7/13
CCR3	+	-	-	ND	CCL11
CCR4	+	-	+	ND	CCL17/22
CCR5	+	-	+	-	CCL3/4/5
CCR6	+	-	-	ND	CCL20
CCR7	-	+	+	+	CCL19/21
CCR8	+	-	-	ND	CCL1
CXCR1	+	-	+	ND	CXCL6/8
CXCR2	+	-	+	ND	CXCL1-3, 5-8
CXCR3	-	-	+	-	CXCL9/10
CXCR4	+	+	+	-	CXCL12
CXCR5	-	+	-	ND	CXCL13

This table is adapted from Cavanagh et al (Cavanagh and Von Andrian, 2002) and modified according to the references (Sozzani, 2005) (Penna et al., 2002) (Saeki et al., 2000). ND, not determined; MDC, monocyte-derived dendritic cell; PDC, plasmacytoid dendritic cell.

Most of the chemokines expressed by immature DC are inducible upon infection. Immature DC then populate the tissue and migrate towards these chemokines, thus accumulating at the site of infection. This is a very important mechanism for enhancing antigen uptake by immature DC during the inflammatory reaction (McColl, 2002). Furthermore, it has been shown that different immature DC subsets display unique sensitivity to certain chemokines, which allows them to be recruited to various peripheral tissues where they play a sentinel role. For instance, a previous study has reported that F4/80-B220-CD11c+ DC expressing CCR1 and CCR5 are mobilized rapidly into the circulation in mice injected with *Propionibacterium acnes* to induce granulomas in the liver, and are recruited into inflammatory tissue by CCL3/MIP-1α, which binds to CCR1 and CCR5 (Yoneyama et al., 2001). CCL20/MIP-3α, produced by keratinocytes, is important in recruiting Langerhans cells (skin dendritic cells) to the epidermis through CCR6 (Charbonnier et al., 1999). Recent data indicate that the ligand for CCR6, CCL20 also is expressed in intestinal Peyer's patches (McColl, 2002), and can be induced in human intestinal epithelium by *Salmonella* infection (Izadpanah et al., 2001). Furthermore, *Salmonella* flagellin has been identified to be responsible for this CCL20-mediated migration of DC (Sierro et al., 2001). These observations confirm that the CCR6/CCL20 axis also plays an important role in migration of DC within the peripheral tissues, especially in the skin and mucosa.

Under inflammatory conditions, an array of chemokines are expressed by resident cells and presented by activated endothelial cells. These include CCL2, CCL5, CCL7, CCL13, CCL20 and CCL22 (Rot and von Andrian, 2004). In

addition, pro-inflammatory mediators, such as TNF-α, and IL-1 produced by tissue macrophages will further contribute to the expression of inducible chemokines by virtually all cells resident in the area (Sozzani, 2005). As mentioned above, immature DC express a panel of chemokine receptors that can interact with these inflammatory chemokines. Following antigen acquisition and processing, DC undergo the process of maturation, which is associated with a "switch" in chemokine receptor expression (Fleming et al., 2003; Ohl et al., 2004; Yanagawa and Onoe, 2003). This involves loss of surface expression of inflammatory chemokine receptors such as CCR6, CCR1, CCR2 and CCR5 and the ability to respond to their ligands (Sozzani et al., 1999; Vecchi et al., 1999). At the same time, they primarily gain surface expression of CCR7 and the ability to respond to its ligands CCL19 and CCL21 (Sallusto et al., 1998). It appears that both the downregulation of inflammatory chemokine receptors and up-regulation of CCR7 are required for migration of mature DC to draining lymph nodes, because the persistent production of inflammatory chemokine by local inflammatory milieu would retain mature DC in the periphery. Mature DC may also express other chemokine receptors apart from CCR7, such as CXCR4 and CXCR5. The former is known to regulate homeostatic trafficking of leukocytes (Sallusto et al., 1998) and the latter is found to regulate the DC migration to B cell zone in lymph nodes (Kim, 2005; Saeki et al., 2000). These chemokine receptors expressed on DC may be involved in the persistent interactions between DC and T and B cells after initiation of immune response (Cyster, 2005).

Increasing data have demonstrated the importance of the axis of CCR7/CCL19 and CCR7/CCL21 in guiding mature DC migration to SLO (Forster et al., 1999; Martln-Fontecha et al., 2003; Ohl et al., 2004). Firstly, constitutive expression of CCL19 and CCL21 in the T cell zone in the draining lymph nodes (Luther et al., 2000; Willimann et al., 1998) and by high endothelial venules lining the draining lymphatics (Saeki et al., 1999) have been demonstrated. Study of naturally occurring CCL19 and CCL21 mutant mice (*plt/plt*) show that DC in these mice fail to migrate from peripheral tissues to draining lymph nodes under either normal conditions or during an immune response (Gunn et al., 1999; Nakano and Gunn, 2001; Stein et al., 2000). In addition, treatment of mice with neutralising anti-CCL21 antibodies prevents migration of antigen-loaded mature DC from ventral skin to inguinal lymph nodes (Saeki et al., 1999). Similarly, CCR7 deficient mice also show impaired migration of mature DC to draining lymph nodes under either steady-state or inflammatory conditions (Ohl et al., 2004). CCR7 mediated steady-state DC migration has been thought to be critical for DC to maintain peripheral tolerance, whereas migration to the T cell area of secondary lymph organs under inflammatory condition is the prerequisite for mature DC to stimulate clonal expansion of naive T cells.

Besides responding to chemokines through regulation of chemokine receptor expression, numerous studies have shown that DC also produce a wide range of chemokines themselves. Upon stimulation in tissue, immature DC produce inflammatory chemokines including CXCL8, CXCL10, CCL3, CCL4 and CCL5, which presumably further enhance recruitment of neutrophils and monocytes to the site of infection (Foti et al., 1999; Padovan et al., 2002; Sallusto et al., 1999;

Yoneyama et al., 2002). As discussed earlier, upon maturation, DC down-regulate the cognate receptors of an array of inflammatory chemokines, such as CCR2, CCR3, CCR5 and CCR6 (Table 1.2). It has been suggested that by producing inflammatory chemokines, the corresponding receptors are desensitised. This mechanism may potentially further reduce the responsiveness of DC to these chemokines, therefore enhancing migration of mature DC from peripheral tissues toward secondary lymphoid tissue.

As DC mature and migrate to the SLO, there is another "switch" in chemokine production. DC lose the capacity to produce those chemokines such as CXCL8, CXCL10, CCL3, CCL4 and CCL5, and begin to produce another set of chemokines, which include CCL17, CCL18, CCL19 and CCL22 (Imai et al., 1999; Sallusto et al., 1999; Vulcano et al., 2001). These chemokines function to attract T and B lymphocytes. The ability of DC to produce chemokines and switch their production facilitates the generation of appropriate immune response required for eliminating pathogens.

1.9 The interaction between chemokines and macrophages

Compared to DC, there are limited studies with respect to interaction between chemokines and macrophages. As an important component of innate immune system, macrophages play a critical role in first line defence against microbes, especially in the initiation of inflammatory responses. During the course of inflammation, macrophages are known to be a major source of inflammatory chemokines. Since rheumatoid arthritis is a chronic inflammatory disease associated with a massive infiltration of inflammatory cells, especially

macrophages, in the synovium of multiple joints (Nanki et al., 2004), the interaction between chemokines and macrophages has been extensively studied in this setting. Macrophages in synovial tissues of rheumatoid arthritis have been shown to constitutively produce a group of chemokines including CXCL8 (Koch et al., 1991), CXCL9 (Konig et al., 2000; Patel et al., 2001), CCL20 (Page et al., 2002), CCL5 (Volin et al., 1998) and CX_3CL1 (Ruth et al., 2001). LPS has been suggested to be the major inducer of these chemokines in the synovial tissues in ReA (Gaston et al., 1999). Most of these chemokines are potent chemotactic factors for a variety of cell types including neutrophils, monocytes and T lymphocytes, in addition, the expression of these chemokines can be elevated by the production of inflammatory cytokines such as IL-1, TNF-α, IL-17 and IL-18 (Chabaud et al., 2001; Matsui et al., 2001).

Furthermore, as the precursor for tissue macrophages, monocytes also express a wide range of chemokine receptors, including CCR1, CCR2, CCR3, CCR4, CCR5 and CXCR4 (Fantuzzi et al., 1999; Katschke et al., 2001; Sica et al., 1997), through which monocytes are continuously recruited to the inflamed peripheral tissues and differentiate into tissue resident macrophages. CCR2 and CCR3 are downregulated during differentiation to macrophages, whereas the expression of CCR1, CCR4, CCR5 and CXCR4 remains high in macrophages (Fantuzzi et al., 1999). These chemokine receptors are thought to play an important role in maintaining activated macrophages at the sites of inflammation.

Thus, macrophages produce a large number of inflammatory chemokines in the inflamed peripheral tissues, and macrophages and their precursors respond to these inflammatory chemokines and so are recruited into the inflamed areas from the blood stream.

1.10 Aim of the project

ReA is an immune-mediated synovitis resulting from persistence of non-culturable bacteria or immunogenetic bacterial antigens in the joints, and HLA-B27 is a strong genetic predisposing factor in the development of ReA (Colmegna et al., 2004). Many studies have focused on the pathogenetic roles of aberrant forms of HLA-B27 molecules, which favour the notion that ReA is an autoimmune disease. However, studies on the persistence of bacterial antigens, T-cell responses and cytokines also indicate the essential contribution of these factors in the pathogenesis of ReA. Bacterial antigens have been identified in the joints of patients with ReA. As the infection initiates at mucousal surfaces, it has been suspected that bacterial antigens are transported to the joints from the original infection site, possibly by monocytes and/or macrophages. However, no study to date has provided direct evidence for this hypothesis.

Salmonella is one of the most common triggering bacteria of ReA. The interactions between *S. typhimurium* and DC or *S. typhimurium* and macrophages have been extensively studied with respect to DC and macrophage activation and subsequent antigen presentation to different T cell subsets. However, the *in vivo* behaviour of these cells upon *Salmonella*

infection, and potential dissemination of bacteria by the cells remains largely unknown.

The aim of this project was to investigate the *in vivo* behaviour of *Salmonella*-infected macrophages and DC, utilising an HLA-B27 transgenic mouse model. The migration pattern of infected cells was analysed, and the role of specific chemokine receptors investigated. In addition, TCR transgenic mice, in which T cells are specific for a *Salmonella*-expressed, HLA-B27-restricted antigen, were employed to examine the capacity for infected APCs to present antigens to CD4+ and CD8+ T cells.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Biochemical reagents

Reagent	Catalogue Number	Supplier
Iscove's Modified Dulbecco's Medium (IMDM)	31980-022	Gibco
Dulbecco's Modified Eagle Medium (DMEM)	31885-023	Gibco
RPMI 1640 Medium with glutamine	21875-034	Gibco
L-glutamine for DMEM medium	25033-010	Gibco
Foetal Bovine Serum for Mac	318A	Autogenbioclear
Foetal Calf Serum for DC (Batch No. 3011101)	S-0001E	Harlan
EDTA	E-7889	Sigma
DMSO	D8779	Sigma
Sodium Azide (NaN3)	S8032	Sigma
Recombinant murine GM-CSF	415-ML	R&D Systems
Recombinant murine GM-CSF	ABC033	Autogenbioclear
LPS from *S. typhimurium* SL1181	L9516	Sigma
IL-2	402-ML-100	R&D Systems
NP383 peptide	n/a	IAH
NP366 peptide	n/a	IAH
BG876 peptide	n/a	IAH
Recombinant mouse MIP3α (CCL20)	760-M3	R&D Systems
Recombinant mouse MIP3β (CCL19)	440-M3/CF	R&D Systems
2-Mercaptoethanol	M7522	Sigma
Sodium Pyruvate	11360-039	Gibco
Ampicillin	A9393	Sigma
Penicillin/Streptomycin	115140-122	Gibco
Gentamicin Solution	G1397	Sigma
Accutase	A6964	Sigma
Phosphate Buffered Saline (PBS)	14190-144	Gibco
Bovine Serum Albumin (BSA)	A4503	Sigma
Trypan Blue	T1854	Sigma
NycoPrepTM A	1002380	AXIS-SHIELD
Sodium Azide	S8032	Sigma
Paraformaldehyde	P6148	Sigma
Brefeldin-A	B-7651	Sigma
Saponin	S-4521	Sigma
Collagenase III	4183	Worthington
Dnase I	DN-25	Sigma
VybrantTM CFDA SE Cell Tracer Kit	V-12883	Molecular Probes
CellTracker Orange CMTMR	C-2927	Molecular Probes
Betaplate Scint	SC/9200/21	PerkinElmer

2.1.2 Fluorescence activated cell sorting (FACS) antibodies

Antigen	Source species	Con-jugate	Isotype	Clone	Final con-centration	Catalogue number	Supplier
Mouse F4/80	Rat	FITC	IgG2b	CI:A3-1	1:10 D**	MCA497F	Serotec
Mouse CD54	AmH*	FITC	IgG1	3 E 2	5 µg/ml	553252	Pharmingen
Human HLA-B27	Mouse	FITC	IgG2a	HLA-ABC-m3	1:10 D	MCA116F	Serotec
I-Ad/Ed	Rat	FITC	IgG2a	2G9	2.5 µg/ml	06344D	Pharmingen
Mouse H-2Dd	Mouse	FITC	IgG2a	34-2-12	5 µg/ml	06134D	Pharmingen
Mouse CD11b	Rat	FITC	IgG2b	M1/70	5 µg/ml	01714D	Pharmingen
Mouse CD11c	AmH	FITC	IgG1	HL3	5 µg/ml	553801	Pharmingen
Mouse Gr-1	Rat	FITC	IgG2b	RB6-8C5	5 µg/ml	01214D	Pharmingen
Mouse CD40	Rat	FITC	IgG2a	3/ 2 3	5 µg/ml	09404D	Pharmingen
Human TCR Vβ7	Mouse	FITC	IgG2a	Zoe	1:10 D	MCA1594F	Serotec
Mouse F4/80	Rat	PE	IgG2b	CI:A3-1	1:10 D	MCA497PE	Serotec
Mouse CD11c	AmH	PE	IgG1	HL3	2 µg/ml	557401	Pharmingen
Mouse IL-6	Rat	PE	IgG1	MP5-20F3	2 µg/ml	01345A	Pharmingen
Mouse IL-12(P40)	Rat	PE	IgG1	C15.6	2 µg/ml	18495A	Pharmingen
Mouse H-2Kd	Mouse	PE	IgG2a	SF1-1.1	2 µg/ml	553566	Pharmingen
I-A/E	Rat	PE	IgG2b	M5/114.15.2	0.5 µg/ml	557000	Pharmingen
Mouse B220	Rat	PE	IgG2a	RA3-6B2	2 µg/ml	01125B	Pharmingen
Mouse CD86	Rat	PE	IgG2a	GL1	2 µg/ml	09275B	Pharmingen
HumanTCRVβ7	Mouse	RPE	IgG2a	Zoe	1:10 D	MCA1594PE	Serotec
Mouse F4/80	Rat	Biotin	IgG2b	CI:A3-1	10 µg/ml	MCA497B	Serotec
Mouse CD80	AmH	Biotin	IgG	16-10A1	5 µg/ml	09602D	Pharmingen
Mouse CD86	Rat	Biotin	IgG2a	GL1	5 µg/ml	09272D	Pharmingen
Mouse CD40	Rat	Biotin	IgG2a	3/ 2 3	2 µg/ml	553789	Pharmingen
Mouse H-2Dd	Mouse	Biotin	IgG2a	34-2-12	5 µg/ml	553578	Pharmingen
Mouse I-Ad/I-Ed	Rat	Biotin	IgG2a	2G9	10 µg/ml	06342D	Pharmingen
Mouse CD11c	AmH	Biotin	IgG1	HL3	5 µg/ml	09702D	Pharmingen
Mouse CD11b	Rat	Biotin	IgG2b	M1/70	5 µg/ml	01712D	Pharmingen
Mouse CD3	AmH	Biotin	IgG	145-2C11	5 µg/ml	553059	Pharmingen
Mouse CD11c	AmH	APC	IgG1	HL3	2 µg/ml	550261	Pharmingen
Mouse TNF-α	Rat	APC	IgG1	MP6-XT22	4 µg/ml	554420	Pharmingen
Mouse IFN-γ	Rat	APC	IgG1	XMG1.2	4 µg/ml	554413	Pharmingen
Mouse CD8a	Rat	APC	IgG2a	53-6.7	1 µg/ml	553035	Pharmingen
Human HLA-ABC	Mouse	APC	IgG1	G46-2.6	1:2.5 D	555555	Pharmingen
Mouse CD4	Rat	Cychrome	IgG2a	RM4-5	1 µg/ml	553050	Pharmingen
Annexin V	n/a	Cy5	n/a	n/a	1:20 D	559934	Pharmingen
MouseCD16/CD32	Rat	Purified	IgG2b	2.4G2	1 µg/ml	01241D	Pharmingen
Streptavidin	n/a	PE-Cy5	n/a	streptavidin	0.4 µg/ml	554062	Pharmingen

* AemH=American Hamster; **D=dilution

2.1.3 FACS Isotype controls

Isotype	Source species	Conjugate	Clone	Final concentration	Catalogue number	Supplier
IgG1	ArmH	FITC	G235-1	5 µg/ml	11134C	Pharmingen
IgG2a	Rat	FITC	R35-95	5 µg/ml	553929	Pharmingen
IgG2b	Rat	FITC	A95-1	5 µg/ml	553988	Pharmingen
IgG2a	Mouse	FITC	G155-178	5 µg/ml	553456	Pharmingen
IgG2a	Mouse	PE	n/a	1:10 D	MCA929PE	Serotec
IgG1	Rat	PE	R3-34	5 µg/ml	20615A	Pharmingen
IgG2a	Rat	PE	R35-95	5 µg/ml	553930	Pharmingen
IgG2b	Rat	PE	A95-1	5 µg/ml	20185A	Pharmingen
IgG2b	Rat	Biotin	A95-2	5 µg/ml	553987	Pharmingen
IgG2a	Rat	APC	R35-95	5 µg/ml	553932	Pharmingen
IgG2b	Rat	APC	A95-1	5 µg/ml	556924	Pharmingen
IgG1	Mouse	APC	MOPC-21	1:2.5 D	555751	Pharmingen
IgG2a	Rat	Cychrome	R35-95	5 µg/ml	553931	Pharmingen

2.1.4 Molecular biological reagents

Reagent	Catalogue Number	Supplier
RNeasy Mini Kit	74103	Qiagen
Rnase-free Dnase	18068-015	Gibco Invitrogen
Oligo$(dT)_{12-18}$ Primer	18418-012	Gibco Invitrogen
Reverse Transcriptase	12328-019	Gibco Invitrogen
Rnasin Ribonuclease Inhibitor	N2111	Promega
Dithiothreitol (DTT)	Y00147	Gibco Invitrogen
Deoxynucleoside triphosphates (dNTPs)	U1240	Promega
BIOTAQ™DNA polymerase	BIO-21040	Bioline
Reverse Transcriptase qPCR™ Master Mix	RT-QPRT-032X	Eurogentec
Agarose electrophoresis grade	15510-027	Invitrogen
Gel loading buffer	G-7654	Sigma
Ethdium bromide	E-1510	Sigma
1Kb DNA ladder	15615-016	GibcoBRL
phiX174 DNA/Hae III Markers	G1761	Promega
DEPC water	9906	Ambion
1Kb plus DNA ladder (1ug/ul)	10787-018	Invitrogen

2.1.5 Immunohistochemistry reagents

Antigen	Source species	Conjugate	Isotype	Clone	Final concentration	Catalogue number	Supplier
Mouse F4/80	Rat	Purified	IgG	Cl:A3-1	10 µg/ml	MCAP497	Serotec
Mouse F4/80	Rat	Biotin	IgG2b	Cl:A3-1	10 µg/ml	MCA497B	Serotec
Mouse CD11c	ArmH	Biotin	IgG1	HL3	5 µg/ml	09702D	Pharmingen
Anti-FITC	Rabbit	Purified	n/a	Polyclonal	1:1000 D	V0403	Dako
Salmonella O antiserum	Rabbit	Purified	n/a	Polyclonal	1:100 D	240984	BD
Anti-rat IgG	Rabbit	Biotin	IgG	Polyclonal	15 µg/ml	BA-4001	Vector
Anti-rabbit IgG	goat	Biotin	IgG	Polyclonal	5 µg/ml	BA-1000	Vector

2.2.6 Immunohistochemistry auxiliary reagents

Reagent	Catalogue number	Supplier
Normal horse serum	S-2000	Vector
Normal goat serum	S-1000	Vector
Rabbit IgG	I-1000	Vector
Avidin/Biotin blocking kit	SP-2001	Vector
Antigen unmasking solution	H-3300	Vector
ABC reagent kit	PK-6100	Vector
NovaRED substrate kit for peroxidase	SK-4800	Vector
Diff-Quik staining set	130832	Dade Behring
Absolute Ethanol	10107	BDH, UK
Acetone	100034Q	BDH, UK
30% H_2O_2	H1009	Sigma
Haematoxyline	H-3401	Vector
Histoclear	H/0468/017	Fisher Chemicals
VectorMount Mounting medium	H-5000	Vector
DPX	08600E	Surgipath Europe Ltd
2-Methylbutane	59080	Fluka
Tissue-YEK O.C.T. Compound	4583	Bayer

2.1.7 ELISA & ELISpot antibodies

Reagent	Clone	Final con-centration	Catalogue Number	Supplier
Purified anti-M-CSF(CSF-1)	5A1	1 µg/ml	552513	Pharmingen
Biotinylated anti-M-CSF	D24	5 µg/ml	552514	Pharmingen
Purified anti-IFN-γ	R4-6A2	10 µg/ml	551216	Pharmingen
Biotinylated anti-IFN-γ	XMG1.2	2 µg/ml	554410	Pharmingen

2.1.8 ELISA & ELISpot reagents

Reagent	Catalogue Number	Supplier
Recombinant mouse M-CSF	ABC 225	Autogenbioclear
Streptavidin HRP conjugate	554066	Pharmingen
Marvel dried skimmed milk	n/a	n/a
Tween 20	P-1379	Sigma
TMB substrate	52-00-00	Sure Blue
Methanol	101586B	Sigma
Dimethylformamide	D8654	Sigma
AEC substrate	A6926	Sigma
Acetic acid	420-10	Sigma
Sodium acetate	102364Q	BDH
$NaHCO_3$	S-6014	Sigma
Na_2CO_3	S-6139	Sigma

2.1.9 Griess Assay reagents

Reagent	Catalogue Number	Supplier
Sodium Nitrite	S2252	Sigma
Sulphanilamide	S9251	Sigma
Napthylethylenediamine (NAP)	N9125	Sigma
Phosphoric acid	P6560	Sigma

2.2.10 Limulus Amebocyte Lysate (LAL)Assay reagents

Reagent	Catalogue Number	Supplier
LPS	E110	Charles River Laboratories
LAL reagent	R19000P	Charles River Laboratories

2.1.11 Bacterial Strains

Strain	Characteristics	Reference
SL3261	aroA-auxotropic non-virulent mutant of *S. typhimurium*	(Hoiseth and Stocker, 1981)
waaN	*S. typhimurium* mutant with depletion-insertion in waaN gene	(Khan et al., 1998)
C5 (TS)	Temperature sensitive (TS) mutant from *S. typhimurium* C5, optimal growth temperature is 30°C	(Hormaeche et al., 1981)
Lb5010	Semi-rough and poorly invasive strain of *S. typhimurium*	(Bullas and Ryu, 1983)

2.1.12 Transgenic Mice

Mouse	Background	Reference
HLA-B2705/human β2M Homozygote	BALB/C	(Weiss et al., 1990)
GRb TCR transgenic mice	HLA-B27/b2M	(Roddis et al., 2004)

2.1.13 Primers for PCR

Name	Sequence		Product size(bp)	Producer	Reference
β-actin	Forward	5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'	400	Sigma-Genosys	(Cheminay et al., 2002)
	Reverse	5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'			
CCR7	Forward	5'-CAT CAG CAT TGA CCG CTA CGT-3'	297	Sigma-Genosys	(Cheminay et al., 2002)
	Reverse	5'-TAC GGA TGA TAA TGA GGT AGC A-3'			
CCR6	Forward	5'-ACT CTT TGT CCT CAC CCT ACC G-3'	300	Sigma-Genosys	Designed by myself
	Reverse	5'-ATC CTG CAG CTC GTA TTT CTT G-3'			

2.1.14 Primers and probes used in real-time PCR

Name	Sequence		Producer
28SRNA	Forward	5'-CGCCGCTAGAGGTGAAATTCT-3'	Sigma-Genosys
	Reverse	5'-CATTCTTGGCAAATGCTTTCG-3'	Sigma-Genosys
	Probe	FAM-ACCGGCGCAAGACGCACCAG-TAMRA	Eurogentec
CCR7	Forward	5'-CCCAAAACGACAGCCAAAA-3'	Sigma-Genosys
	Reverse	5'-GGCCCCACATCCCTCACT-3'	Sigma-Genosys
	Probe	FAM-AAAGTGAGAGGCTGCCACACTTTCCG-TAMRA	Eurogentec
CCR6	Forward	5'-TTGGTGCAGGCCCAGAA-3'	Sigma-Genosys
	Reverse	5'-CACGAGAACCACAGCGATCA-3'	Sigma-Genosys
	Probe	FAM-CCAAGAGGCACAGAGCAATCCGAGTC-TAMRA	Eurogentec

2.1.15 Equipment

Item	Model	Supplier
Dual Laser FACScaliber Flow Cytometer	n/a	Becton Dickinson, UK
GeneAmp 9700 PCR Thermal System	4314879	Perkin-Elmer
TCS-NT Confocal Microscope	n/a	Leica, UK
Water Jacketed, Heated, CO_2 Incubator	3111	Forma Scientific, UK
1450 Microbeta Trilux Scintillation Counter	1450-028	Wallac, UK
ABI prism 7700 Sequence Detector	7700	PE Applied Biosystems
Microtiter Plate Reader	SpectraMax 340	Molecular Devices
Cytospin	Cytospins 3	Shandon
Cryostat	CM1900	Leica
Alpha Imager Digital Imaging System 2200	n/a	Alpha Innotech, UK
96-well Automatic Plate Washer	Skanwasher 300	Skantron, US
Gamma Cell Irradiator (Caesium^{137})	Gamma cell Elite	Nordion
GeneQuant pro RNA/DNA Calculator	N/a	Amersham pharmacia biotech
Walker Safty Cabinet	Model 1.2	Holten LaminAir
GS-15R Centrifuge	GS-15R	Beckman
GS-6KR Centrifuge	GS-6KR	Beckman
GS-6R Centrifuge	GS-6R	Beckman
Microfuge®R Centrifuge	F241.5	Beckman
Clifton Unstirred Bath	n/a	Clifton
Heidolph Incubator 1000	1000	Heidolph
Heidolph Unimax 1010	1010	Heidolph
Slide Hybridisation/Incubation Chamber	INH-110-010H	Fish Scientific UK
uMACS Separator Unit	130-042-602	Miltenyi Biotec

2.1.16 Plasticware and sundries

Item	Catalogue Number	Supplier
Haemocytometer	Hae2000	Scientific Laboratory Supplies
50 ml Centrifuge Tubes	3041016	BD Falcon
15 ml Centrifuge Tubes	4-2096-2	BD Falcon
90 mm Petridish	1029	BD Falcon
Terumo Needles 27 gx3/4"	NN-2719R	Terumo
Terumo Needles 25 gx5/8"	NN-2516R	Terumo
T125 Tissue Culture Flask	90151	TPP
T75 Tissue Culture Flask	90076	TPP
T25 Tissue Culture Flask	9025	TPP
1 ml Micro Tubes (FACS)	B613	Scientific Laboratory Supplies
5 ml Polypropylene Tubes (FACS)	2054	BD Falcon
6-well Plate	140685	NUNC
24-well Plate	146485	NUNC
96-well Plate	163320	NUNC
Syringe Driven Fliter Unit (0.22um)	SLGP033RS	Millipore
500 ml 0.22 μm Filter Bottle	TKV-246-021U	Nalgene
Multiscreen IP Plates for ELISpots	ELIIP10SSP	Millipore
ELI-Puncher Kit	Melipunch	Millipore
96-well Maxi-Sorp Immuno Plate	442404	NUNC
Thermo-Fast®96, Non-Skirted PCR Tube	AB-0600/G	Abgene, UK
ThermoFast®96, Semi-skirted PCR Tube	AB-0900	Abgene, UK
Domed Cap Strip	AB-0265	Abgene, UK
24-well Transwell Plate (5 μm pore size)	3421	Corning Incorporated
Plate Sealer	FIL1080	Sicentific Laboratory Supplies
Superfrost "plus" White Slides (for IHC staining)	08143G	Surgipath Europe Ltd
Frosted Microscope Slides (for mashing tissue)	2951	Esco
CryoTube Vials	368632	Sicentific laboratory supplies
MACS Separation Column (LS)	130-042-401	Miltenyi Biotec
MACS Separation Column (MS)	130-042-201	Miltenyi Biotec
Glass Fibre Filter	1450-421	Wallac
Sample Bag	1450-432	PerkinElmer

2.2 Methods

2.2.1 Animals

All mice were maintained in the specific pathogen-free facility at the Institute for Animal Health (Compton, U.K.).

HLA-B27/β_2m (BALB/c background) mice, expressing HLA-B2705 and human β_2m were originally obtained from E. Weiss (Weiss et al., 1990) and were inbred as a homozygous inbred line. The GRb transgenic line, expressing both the α- and β-chains of the GRb TCR, was maintained as a heterozygous line backcrossed to B27/β_2m mice (GRb × B27/b_2m) (Roddis et al., 2004).

All animal experiments were carried out under a Home Office project license in compliance with relevant laws and local guidelines and were approved by the Institute for Animal Health ethical committee.

2.2.2 Cell culture

2.2.2.1 Maintenance of Ladmac cell line

To supply macrophage-colony stimulating factor (M-CSF, also named CSF-1) for growing BMMac, the Ladmac cell line, a cell line secreting the growth factor M-CSF, was obtained as a gift from Dr. Sam Hou in the Edward Jenner Institute for Vaccine Research. This cell line was originally made by transfecting mouse bone

marrow cells, highly enriched for macrophage progenitors, with a human R-myc oncogene (Sklar et al., 1985).

An aliquot of Ladmac cells was removed from liquid nitrogen and defrosted in a 37°C water bath until a small ice pellet remained. Dulbecco's Modified Eagle Medium (DMEM)/10 (DMEM, 10% FCS, 50 μM β-Mercaptoethanol (ME), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine) was prepared, and 500 μl of DMEM/10 was slowly added to the sample. The sample was transferred to a 15 ml centrifuge tube and a further 5 ml media was added dropwise. After centrifugation at 375 × g for 5 minutes, the cell pellet was resuspended in 10 ml DMEM/10. Cell counting was performed as follows: 10 μl of cell suspension was diluted 1:2 or 1:10 in typan blue depending on the estimated density of the cells. Typan blue diluted cells, in 10 μl, were then dropped onto a haemocytometer and the cells located in the central compartment (25 squares) were counted using a light microscope. The concentration of cell suspension was worked out as the number counted × 10^4/ml. The viability of cells was determined by dividing the number of cells excluding the typan blue dye (indicating live cells) by total number of cells × 100%.

The cell suspension was transferred to a T175 tissue culture flask containing 150 ml DMEM/10 and incubated for 2 weeks at 37°C, 5% CO_2. Following incubation, the cell supernatant was harvested, filtered through a 0.22 μm filter and stored at -20°C in 50 ml aliquots. These aliquots were subsequently used as a source of M-

CSF in the growth of BMMac. The cells remaining adhered to the flask were flushed with 10 ml DMEM (50 μM , 100 U/ml penicillin, 100 μg/ml streptomycin) and the cell suspension was centrifuged at 375 × g for 10 minutes. The supernatant was discarded leaving 1 ml of media in which the cells were resuspended. Cell suspension in 1 ml (approximately 5×10^6) was then added to a new T175 flask along with 150 ml DMEM/10 and the flasks incubated for 2 weeks at 37°C, 5% CO_2 as before.

2.2.2.2 M-CSF Enzyme-Linked Immunosorbent Assay (ELISA)

To quantitate the secretion of M-CSF in the Ladmac cell culture supernatant, an ELISA for M-CSF was used. A 96-well Maxi-Sorp Immuno plate was coated overnight at 4°C with the capture antibody, anti-murine M-CSF monoclonal antibody diluted (final concentration 1 μg/ml) in carbonate buffer (0.045 M $NaHCO_3$, 0.018 M Na_2CO_3, pH 9.6). The plate was blocked for 1 hour at room temperature with 4% marvel in PBS-T (0.05% Tween 20 in PBSa). After washing six times with 150 μl PBS-T, different batches of Ladmac cell culture supernatant (neat or tenfold diluted) were added to the wells in triplicate. Seven two-fold serial dilutions (156-10000 pg/ml) of murine recombinant M-CSF were used to make the standard curve with the no addition wells as negative control. After a two hour incubation at room temperature, the plate was washed with PBS-T. Following this, 100 μl biotinylated rat anti-mouse M-CSF diluted in 1% (w/v) marvel PBS-T (5 μg/ml final concentration) was added as the detection antibody and the plate was incubated for two hours at room temperature followed by PBS-T wash. Horseradish

peroxidase (HRP)-conjugated streptavidin diluted 1:1000 in PBS-T was added to the wells. After washing six times with 150 μl PBS, bound HRP was visualised by addition of 100 μl TMB Microwell Peroxidase/Substrate. The plate was left for 5-10 minutes, the colour reaction stopped by addition of 25 μl 1 M H_2SO_4 (final concentration 0.2 M), and the absorbance of reaction was measured at 450 nm in a microtiter plate reader. The amount of M-CSF in supernatant was calculated according to the standard curve of recombinant M-CSF.

2.2.2.3 Preparation and culture of BMMac

To obtain BMMac, femurs and tibiae were removed from mice sacrificed by an increasing concentration of CO_2. Following soaking in 70% (v/v) ethanol for 30 seconds and air drying, excess fat and tissue were removed and bone marrow was exposed by cutting both ends of the bones with scissors. Bone marrow was flushed out with PBS under sterile conditions using a syringe with a 25 G needle. After pipetting and washing in PBS, a single cell suspension was pelleted and resuspended in 2 ml of red cell lysis buffer (0.15 M NH_4CL, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4). After incubating for 3 minutes on ice, 8 ml of PBS was added and the cell suspension filtered through 40 μm strainer and centrifuged for 5 minutes at 375 × g. The pellet was resuspended in 10 ml of Iscove's Modified Dulbecco's Medium (IMDM)/10 (IMDM, 10% FCS, 50 μM β-ME, 100 U/ml penicillin and 100ug/ml streptomycin). Bone marrow cells (1-3 × 10^7) were aliquoted into a T75 tissue culture flask containing 20 ml of IMDM/10 supplemented with 20% Ladmac cell supernatant (final concentration equivalent to 10 ng/ml recombinant

murine M-CSF as determined by ELISA as detailed in 2.2.2.2). The flask was incubated for 3 weeks at 37°C, 5% CO_2. A further 10 ml IMDM/10 with 20% Ladmac cell supernatant was added every 3 days. The adherent cells were harvested by unidirectionally scraping the surface of the flask with a cell scraper.

2.2.2.4 LAL assay

Different batches of foetal calf serum (FCS) may contain various level of endotoxin, which could mature dendritic cells in culture. In order to choose low-endotoxin FCS for BMDC culture and therefore obtain immature DC, a panel of FCS was screened using the LAL assay. Firstly, three tenfold serial dilutions of standard solutions of endotoxin were prepared for making standard curve (0.5-50 EU/ml with absence of endotoxin wells as negative control). All the FCS samples were tenfold diluted in endotoxin-free water and 10 μl of this dilution was added to each well of a 96-well plate; triplicate were performed for samples and duplicates for the standard curve. The plate was incubated on a preheated (37°C) microtiter plate reader for 10 minutes without shaking. LAL solution was freshly prepared by adding endotoxin-free water to the LAL reagent following the manufacturer's instruction, 100 μl of which was then added to the wells. The cloudier the reaction indicates the greater presence of endotoxin contamination in the samples. No more than 10 minutes after addition of LAL solution, the absorbance of the reaction was measured at 450 nm in a microtiter plate reader. The endotoxin level of samples was calculated according to the standard curve.

2.2.2.5 Preparation and culture of BMDC

BMDC were generated essentially based on a published protocol (Lutz et al., 1999). Bone marrow was prepared as described 2.2.2.3. Approximately 2×10^6 erythocyte-depleted bone marrow cells were cultured in T75 tissue culture flasks or 100 mm petri-dishes. IMDM/10 in 10 ml along with 20 ng/ml of recombinant murine GM-CSF was added and the cells were incubated in a 37°C, 5% CO_2 incubator for 7 days. At day 3 and 6, a further 10 ml of fresh media was supplemented to the culture in the presence of 20 or 10 ng/ml of GM-CSF respectively. The non-adherent cells were harvested by collecting the culture supernatant. The adherent cells were obtained by adding 2 ml of accutase and incubating for 15 minutes at 37°C. Following this, 5 ml of media was added and the cells were harvested (gentle scraping was applied if necessary). Cells were centrifuged for 5 minutes at 375 × g, and the pellet was resuspended in fresh media and used for further experiments.

2.2.2.6 Culture of CTL cell lines

To analyse T cell responses *in vitro*, different CTL lines were used. These cell lines were made by Clare Thompson in our laboratory. In brief, a HLA-B27 mouse was immunised intraperitoneally with 10^7 p.f.u. recombinant vaccinia virus expressing influenza A virus nucleoprotein (NP) and β-galactosidase (β-gal). The mouse was culled 2 weeks after infection, and the spleen was removed and mashed to obtain the splenocytes, 5×10^6 of which were pulsed for 30 minutes at 37°C with 1 μM of either $NP_{383\text{-}391}$ (SRYWAIRTR) peptide in order to obtain $NP_{383\text{-}391}$ CTL or β-$gal_{876\text{-}}$

$_{884}$ (TPHPARIGL) to gain $BG_{876-884}$ CTL. These cells were mixed with 1.5×10^7 original splenocytes and cultured for 10 days in the presence of mouse recombinant IL-2 (final concentration 5 U/ml). For subsequent stimulations, irradiated splenocytes were used as stimulators. For this, a spleen was removed from a HLA-B27 mouse and mashed to obtain the splenocytes, which were then irradiated with 3000 rads γ-irradiation generated from a gamma cell irradiator. Irradiated splenocytes (approximately 1×10^8 cells per spleen) were then washed and resuspended in 2 ml of T-cell media (RPMI 1640, 10% FCS, 50 μM β-ME , 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate), and peptide was added at a final concentration of 1 μM. After 30 minutes incubation at 37°C, the splenocytes were then washed and resuspended into the T-cell media at a final cell density of 2×10^6/ml. $NP_{383-391}$ CTL or $BG_{876-884}$ CTL were harvested at a cell density of 2×10^5/ml in T-cell media. Equal volumes of irradiated splenocytes and CTL cell suspension were mixed and mouse recombinant IL-2 (final concentration 5 U/ml) was added to the culture. The cell mixture was then incubated at 37°C, 5% CO_2 in a flask or a 24-well or 6-well plate depending on the final culture volume. On day 5 and 8, the culture was fed respectively by replacing half volume of culture supernatant with fresh media containing IL-2 (final concentration 5 U/ml) without disturbing cells in the bottom. Every 10 days, the stimulation was repeated.

2.2.3 Infection of cells with S. typhimurium

2.2.3.1 Bacterial strains

There is a panel of attenuated strains of *S. typhimurium* available in our laboratory. SL3261 is an aroA$^-$ mutant of *S. typhimurium* generated by insertion of a transposon in the gene *aroA* (Hoiseth and Stocker, 1981), which renders it virtually non-virulent and allows it to be used as a live vaccine strain. waaN (also known as MsbB) is a mutant with a depletion-insertion in the waaN gene, which encodes the enzyme that catalyses the reaction that completes lipopolysaccharide (LPS) lipid A biosynthesis. This mutant possesses less toxicity of lipid A, the main component of endotoxin (Khan et al., 1998). C5 (TS) is a temperature sensitive (TS) mutant from *S. typhimurium* C5, which optimally grows at 30°C but ceases to grow at 37°C (Hormaeche et al., 1981). Lb5010 is a poorly invasive strain derived from *S. typhimurium* LT2 (Bullas and Ryu, 1983).

We also have recombinant strains generated from SL3261, called SL3261 $NP_{383-391}$, SL3261 $NP_{366-374}$ and SL3261 $BG_{876-884}$, which were made by Michael Wood and Edouard Galyov from Institute for Animal Health. These recombinant strains express the class I restricted epitopes, amino acids 383-391 (restricted by HLA-B27) and amino acids 366-374 (H-D^b restricted) from the influenza virus nucleoprotein or amino acids 876-884 (restricted by H-K^d) from β-galactosidase. They are expressed as part of a fusion protein with the type III secreted protein SopE (*Salmonella* outer proteins E) (Wood et al., 1996). This previously developed system (Russmann et al., 1998) allows CTL epitopes to be efficiently delivered in the cytosol of infected cells via the needle complex of the TTSS of *S. typhimurium* (Galan and Collmer, 1999).

2.2.3.2 Culture conditions of *S. typhimurium*

In order to enhance the efficiency of infection, the logarithmic state of bacteria was chosen to use for infection. To obtain the optimal time point in the course of bacterial growth, the stocks of 4 strains of *S. typhimurium* (SL3261, waaN (MsbB), C5 (TS) and Lb5010) were thawed from a -80°C freezer, and 5 μl bacterial stock was put into 10 ml (1:2000 dilution) Luria-Bertani (LB) broth. When recombinant bacterium was used, LB broth was supplemented with 100 μg/ml ampicillin. After 16 hours incubation at 37°C for SL3261, waaN and Lb5010 and 30°C for C5 (TS), 500 μl of each bacterial culture was transferred into a conical flask containing 50 ml LB broth (1:100 dilution). The diluted bacterial culture was further incubated at 37°C, or for C5 (TS), at 30°C. Every 20 minutes 1 ml of bacterial culture was taken out, and the optical density (OD) at 581 nm was recorded, until the OD readings were steady. A bacterial growth curve was depicted according to the time versus OD values, and the time point (180 minutes) which was the mid logarithmic phase of bacterial growth was chosen for infection. The bacterial culture at that time point was centrifuged 30 minutes at 2150 × g and resuspended at 10^9 cells/ml in PBS. In some experiments, heat-killed bacteria were prepared by incubating bacterial suspensions at 65°C for 40 minutes. Serial bacterial dilutions in 100 μl were plated onto LB agar plates. After overnight incubation at 30°C for C5 (TS) or 37°C for the others, the number of colony forming unit (CFU) was counted and the total number of bacteria was calculated for checking the multiplicity of infection (M.O.I).

2.2.3.3 Infection of cultured cells with *S. typhimurium*

To analyse the interaction of BMMac or BMDC with *S. typhimurium* and the post-infection effects on these two types of cells, the infection was performed as follows. The supernatant of cell culture was discarded, non-adherent cells were removed by washing the flask twice with PBS, and 10 ml of antibiotic free IMDM/10 was added to the flask. The number of non-adherent cells was predetermined at approximately 5×10^6 per flask when the cells reached confluence, which were then infected at various M.O.I (bacteria per cell) at 30°C for C5 (TS) or 37°C for SL3261 for 2 hours. Following this, the flask was washed three times with PBS, and 10 ml IMDM/10 supplemented with 50 μg/ml gentamicin added. In order to kill extracellular bacteria, the flask was incubated at 37°C for 16 hours. Following this, the cells were harvested as described in section 2.2.2.3 or 2.2.2.5, the pellet resuspended in fresh media and the cells used for further experiments.

2.2.3.4 Analysis of infection rate

To investigate the infection rate of cells being infected with *S. typhimurium*, 2×10^5 infected cells in 200 μl were loaded onto the cytoslide device and centrifuged for 5 minutes at 11 × g in a Cytospin. The cell layer on the slide was left to air dry, then fixed and stained with the Diff-Quik staining set. This comprises 3 solutions; Solution 1 is fixative solution containing 0.002 g/L fast green in methanol; Solution 2 is made of 1.1 g/L eosin G in PBS (pH 6.6) and Solution 3 contains 1.22 g/L thiazine dye in PBS (pH 6.6). The slide was submerged 5 times in each stain for 1 second before being rinsed with running water and left to air dry. A small amount of

mounting media was dropped onto the slide and a coverslip was carefully placed on it. The slide was then examined under oil immersion using light microscopy at a magnification of 100 ×. The number of cells that engulfed bacteria within 100 counted cells was recorded, triplicate counts were applied in three randomly chosen fields, and the mean percentage was calculated as the final infection rate. Photographic images were taken using a microscope equipped with a polaroid camera and the images were analysed using Adobe Photoshop software.

2.2.4 Flow cytometry

2.2.4.1 Extracellular staining

Characterisation of cell surface marker expression on the BMMac and BMDC was carried out using flow cytometry. Cells for analysis were aliquoted into FACS tubes or 96-well U-bottomed plates at a concentration of 2×10^5 cells per tube or well, and washed twice with FACS wash buffer (0.1% FCS, 0.01% sodium azide in PBS). In order to block non-specific binding, the cell pellet was resuspended in Fc block (anti-CD16/CD32, 1 μg/ml final concentration) and incubated on ice for 10 minutes. Appropriate first layer antibodies were then added (final concentration shown in Materials 2.1.2) to the tube and cells were incubated on ice in the dark for 30 minutes. Following incubation, cells were washed twice with FACS wash buffer by centrifugation at 375 × g for 3 minutes and the supernatant discarded. Cells stained with directly conjugated antibodies were then fixed by resuspending the pellet in PBS/1% paraformaldehyde (PFA) and kept at 4°C in the dark. Cells

stained with biotinylated antibodies were resuspended in FACS wash buffer containing a streptavidin-conjugated fluorochrome (final concentration 0.4 μg/ml) and incubated on ice in the dark for another 30 minutes. These cells were then washed twice and fixed in PBS/1% PFA. Fluorescent cells were acquired using a FACSCaliber flow cytometer and analysed using WinMDI FACS analysis software.

2.2.4.2 Intracellular cytokine staining

Cytokine production by *S. typhimurium* infected cells was analysed using intracellular cytokine staining. BMMac or BMDC were infected with *S. typhimurium* as described in section 2.2.3.3. After washing three times with PBS, fresh medium containing 50 μg/ml of gentamicin and 10 μg/ml of brefeldin-A was added to the flask. The role of the brefeldin A is to block protein transport from the endoplasmic reticulum to the Golgi apparatus allowing the secreted cytokine to be confined inside the cells and detected with the assistance of a permeabilisation buffer. The cells were then incubated for 6 hours at 37°C, 5% CO_2. When cytokine production from T cells was measured, T cells were stimulated with the corresponding peptide ($NP_{383-391}$ or $NP_{366-374}$ at a final concentration of 1 μM) for 6 hours in total, in the presence of 10 μg/ml of brefeldin-A for the last 3 hours' incubation. Following incubation, 2×10^5 cells were aliquoted into a FACS tube. The cells were stained for surface molecules as described in 2.2.4.1. The stained cells were fixed in PBS/2% PFA for 20 minutes at room temperature. Fixed cells were washed and resuspended into permeabilisation buffer (0.5% (w/v) saponin in FACS wash buffer) for 20 minutes at room temperature. Staining for intracellular cytokines (final

concentration see Material 2.1.2) was performed for 30 minutes at room temperature in 0.5% saponin. The cells were then washed twice with 0.5% saponin followed by another two washes with FACS wash buffer, and the pellet was resuspended in PBS/1%PFA. Cells were acquired using a FACSCaliber flow cytometer and analysed using WinMDI FACS analysis software.

2.2.4.3 Annexin V staining

Cell death of BMDC or BMMac after *S. typhimurium* infection was evaluated using Annexin V satining as a marker of apoptosis. Cells were stained with Annexin V and propidium iodide (PI) according to the manufacturer's protocol. Briefly, the cells were washed twice with cold PBS and then resuspended at a concentration of 1×10^6 cells/ml in 1 × binding buffer. A 100 μl (1×10^5) aliquot of the cell solution was then transferred to a 5 ml FACS tube, and 5 μl of Annexin V (final dilution 1:20) and 5 μl of PI (final concentration 2.5 μg/ml) were added. The cells were gently vortexed and then incubated for 15 minutes at room temperature in the dark. After incubation, 400 μl of 1 × binding buffer was added to each tube and cells were analysed by flow cytometry within one hour. For our experiments, the cells that were apoptotic were those that were Annexin V positive and PI negative.

2.2.5 Griess Assay

To detect the production of reactive nitrogen intermediates such as NO generated from *S. typhimurium* infected cells by L-arginine oxidation pathway, Nitrite (NO_2^-),

the stable end product of L-arginine oxidation by iNOS (Hibbs et al., 1987), was quantitated in the cell culture supernatant by the Griess diazotization reaction. $NaNO_2$ was used to make the standard curve. Briefly, solution 1 (2% sulphanilamide, 5% H_3PO_4 in distilled water) and solution 2 (0.2% napthylethylenediamine in PBS) were prepared and added in a 1:1 ratio to obtain a colourless solution. Following this, standards of sodium nitrite were prepared in PBS by seven two-fold serial dilutions of a 100 μM solution, wells with no $NaNO_2$ were used as negative control. A 75 μl aliquot of supernatant or standard to be tested was added to the wells of a 96-well MicroTest III plate in triplicate, and a 25 μl of the colourless solution mixture was then added to each test well. The plate was incubated with shaking for 5 minutes on a plate shaker, the absorbance of color reaction was measured at 540 nm wavelength in a microtiter plate reader.

2.2.6 Immunohistochemistry staining

Tissues from mice were mounted in Tissue-Tek O.C.T. Compound, then snap-frozen in a bath of 3-methyl-butane and dry ice. The tissue was cut in 4 μm sections on a cryostat, and kept at 4°C until required. Each section was fixed with acetone for 10 minutes, rehydrated in PBSa and blocked with 10% normal goat serum for 20 minutes followed by 10 minutes incubation with 0.3% H_2O_2 in 10% normal goat serum to quench the endogenous peroxidase activity. For three layered staining, take F4/80 as an example, the section was firstly incubated with the purified rat anti-mouse F4/80 antibody (final concentration 10 μg/ml) at room

temperature for 1 hour, followed by five washes of 5 minutes each with PBSa. Then the section was incubated with the second antibody, biotinylated anti-rat IgG (final concentration 15 μg/ml) at room temperature for 30 minutes followed by five washes with PBSa. The section was incubated with Vectastain ABC mixed reagent for 30 minutes, which was made by mixing 2 drops of Reagent A and 2 drops of Reagent B in 5 ml PBS and standing for 30 minutes at room temperature before use. Following this, the slide was washed with PBSa and the enzyme reaction developed with peroxidase substrate kit following the manufacturer's instructions. Briefly, the solution was made by mixing 3 drops of Reagent 1, 2 drops of Reagent 2 and 2 drops of hydrogen peroxidase solution in 5 ml distilled water. The staining was monitored visually under the microscope until the desired intensity was developed. For two layered staining, after 10% goat serum blocking and incubation with 0.3% H_2O_2, the section was incubated with a biotin conjugated antibody for 30 minutes. After PBSa washes, the section was then incubated with Vectastain ABC mixed reagent. The following steps were then the same as the three layered staining.

2.2.7 Analysis of message RNA (mRNA)

2.2.7.1 Extraction of mRNA

Total cellular RNA from cells was isolated using the RNeasy Mini kit, which applies a technology that combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. This kit uses a specialised high-salt buffer system that allows up to 100 μg of RNA longer than 200 bases to

bind to the RNasy silica-gel membrane. Samples are first lysed and homogenised in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy mini column where the total RNA binds to the membrane and contaminants are efficiently washed away, high–quality RNA is then eluted in water.

Approximately 1~5 × 10^6 BMMac or BMDC were harvested and lysed by addition of buffer RLT (GITC-containing buffer) (350 μl/sample). To ensure thorough lysis, the cell lysate was pipetted onto a QIAshredder spin column, which was placed in a 2 ml collection tube and the sample was centrifuged for 2 minutes at maximum speed (15000 × g). The column was discarded and 350 μl of 70% ethanol (v/v in Milli-Q® water) was added to the lysate. All the volume of the lysate was then applied to a RNeasy mini column placed in a 2ml collection tube. The tube was centrifuged for 15 seconds at ≥ 8000 × g, the flow-through was discarded and 700 μl of buffer RW1 added to the RNeasy column. The sample was centrifuged for 15 seconds at ≥8000 × g, 500 μl buffer RPE was added to the RNeasy column, followed by 15 seconds centrifugation at ≥ 8000 × g, the flow-through was discarded and another 500 μl of RPE was applied followed with centrifugation. To eliminate any chance of possible buffer RPE carryover, the RNeasy column was placed into a new 2 ml collection tube and centrifuged at full speed for 1 minute. RNA was eluted with 3050 μl of RNase-free water into a new 1.5 ml collection tube

by centrifugation for 1 minute at ≥ 8000 × g. The concentration of total cellular RNA in each sample was measured by the optical density at 260 nm at a GeneQuant pro RNA/DNA calculator (OD260= 40 μg/ml).

2.2.7.2 Digestion of contaminated DNA

To remove any contaminating genomic DNA, the samples were subsequently digested with RNase-free DNase I Kit. RNA (1 μg) was added with 1 U of RNase-free DNase I (I U/μl) in 1:10 diluted 10 × DNase I reaction buffer. The mixture was incubated for 15 minutes at room temperature. DNase I was inactivated by addition of 1:10 diluted 25 mM EDTA solution and the reaction mixture heated for 10 minutes at 65°C. The samples were then stored at –80°C in 10 μl aliquots until further use.

2.2.7.3 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

2.2.7.3.1 Reverse-transcription of RNA into complementary DNA (cDNA)

Reverse transcription (RT) is a process that turns messenger RNA into complementary DNA using the enzyme reverse transcriptase. It was performed by mixing 2 μg of total RNA in 10 μl diethylpyrocarbonate (DEPC)-treated water containing 0.5 μg of oligo $(dT)_{12\text{-}18}$ primer (0.5 μg/μl). This solution was incubated for 10 minutes at 65°C. A 10 μl of solution containing 0.5 μl reverse transcriptase (15 U/μl), 4 μl of 5 × reverse transcriptase buffer, 40 U of rnasin ribonuclease inhibitor (40 U/ μl), 20 mM dithiothreitol and 2 mM deoxyribonucleoside triphosphates (dNTPs) were prepared and added to the 10 μl of RNA and oligo

(dT) mix, and the tube was incubated for 60 minutes at 37°C on a DNA thermal cycler. The tube was then heated to 90°C for 5 minutes, and 180 µl of H_2O was added to the reaction mixture to make a 1:10 dilution of cDNA. Samples were stored at -20°C until further use.

2.2.7.3.2 Amplification of DNA using the Polymerase Chain Reaction (PCR)

The principle of PCR is that the two strands of DNA are separated with a brief heat treatment (denaturation), and, after strand separation, cooling of the DNA (annealing) in the presence of a large excess of the specific two primer DNA oliogonucleotides allows these primers to hybridize to complementary sequences in the two DNA strands. This mixture is then incubated with DNA polymerase and the four deoxyribonucleoside triphosphates so that DNA is synthesised (amplification). The entire cycle is then repeated with the newly synthesised fragments serving as templates in their turn, as the procedure is performed over and over again, the DNA is efficiently amplified.

To amplify the reverse transcribled cDNA, 50 µl of cDNA in section 2.2.7.3.1 was added to 50 µl of a solution consisting of 1 U of Biotaq™DNA polymerase (5 U/ml), 200 µM dNTPs, 200 nM forward and reverse primers, and 5 µl of 10 × PCR buffer. β-actin was used as house keeping gene in the reaction. The primers details are listed in Materials section 2.1.13. PCR amplification was started by an initial denaturation step (5 minutes at 94°C), completed by a final amplification step (7 minutes at 72°C), and the PCR cycles consisting of denaturation (30s at 94°C),

annealing (45s at 60°C) and amplification (60s at 72°C), and the reaction was carried out on a DNA thermal cycler. To amplify the cDNA specific for β-actin, 22 cycles of the PCR reaction were performed, whereas 28 cycles were carried out to detect CCR7 and 35 cycles was used to CCR6. A 20 μl aliquot of PCR reaction product was mixed with 5 μl of 5 × gel loading buffer and subjected to electrophoresis on a 2% agarose gel. PCR products were visualised by staining with 0.1 μg/ml ethdium bromide.

2.2.7.4 Real-time RT-PCR (TaqMan)

To quantitate the expression of CCR7 and CCR6 at the RNA level before and after *S. typhimurium* infection, the samples were subjected to real-time RT-PCR analysis by using Reverse Transcripptase qPCR™ Master Mix kit with 28S rRNA used as endogenous control.

The sequence of the primers and probes were designed by PrimerExpress™1.0 (Applied Biosystems) by input of the cDNA sequence of targeting probe, which ideally should contain intron and exon splice junctions in order to avoid the disturbance of the genomic DNA in the reaction. Then the desired primers and probe were chosen manually from the subsequently generated pool of candidates following the rules that primers must not have more than 2 C/G in last 5 nucleotides at the 3 end and if possible the two primers have equal Tms (melting temperatures). As for the probes, they must not start with a G at the 5 end, C≥ G, and the Tm of probe must be 7-10°C higher than its corresponding primers.

Real-time PCR is quantitative RT-PCR, which relies on the fluorescence resonance energy transfer (FRET) for quantification. For doing this, the probe is designed to hybridize to an internal region of a PCR product and is labelled with a fluorescent dye on the 5' base (FAM) and a quenching dye on the 3' base (TAMRA). When stimulated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. During PCR, when the polymerase replicates a template on which a probe is bound, the 5' exonuclease activity of the polymerase cleases the probe. This separates the fluorescent and quenching dyes and FRET no longer occurs. Fluorescence increases in each cycle as the reactions proceeds. All the primers and probes used in assay are listed in Materials section 2.1.14.

Total RNA were extracted from samples following the protocol described in 2.2.7.1. A master mix in 20 μl per well containing 0.125 μl reverse transcriptase (50 U/μl), 12.5 μl 2 × reaction buffer, 0.6 μM primer mix (CCR6 was 0.1 μM) and 0.1 μM probe in DEPC-treated H_2O was prepared firstly. RNA in 5 μl was 1:10 (28S RNA was 1:50) diluted and added to each well in Thermo-Fast®96, non-skirted PCR tube and each sample was performed in triplicate. LPS treated BMDC checked by RT-PCR was used for a CCR7 standard curve and the RNA isolated from spleen cells was used for a CCR6 standard curve. Both were optimised in preliminary experiments. Negative control reactions had no added RNA in the master mix to confirm that there was no non-specific reaction in the assay. The plate was then

loaded onto the ABI PRISM™ 7700 Sequence Detector. Reaction conditions consisted of 30 minutes at 48°C and 10 minutes at 95°C set up for reverse transcription reaction, and the amplification profile was established as 40 cycles of 15 seconds denaturation at 95°C and 1 minute annealing and extension at 60°C for cDNA.

To analyse the data generated from the assay, a second set of PCR reactions was performed for 28S RNA on both the standard curve and experimental samples. Relative abundance values of experimental samples were then normalised by the values of their 28S RNA. Thereafter, different samples could be directly compared according to their normalised values and the fold change among samples calculated. In detail, raw data expressed by circle of threshold (Ct) values for each standard curve were firstly taken to plot a standard curve displayed as a scatter graph with an insertion of a trendline and corresponding equation and R^2 value. Then the Ct values of each probe were calculated by averaging the triplicate values, and standard error (SE) values were obtained subsequently. Following this, for test probes, 40-Ct values were calculated and used in the correction formula. For 28S RNA, an overall mean (and SE) of Ct values were worked out for all the samples, and then the Difference Factor (DF) for each sample was calculated by the following formula:

$$DF = \frac{\text{mean 28S of sample}}{\text{mean overall 28S}}$$

Then, the value of all the samples were corrected as below:

$$\text{Corrected Value} = \frac{[(40\text{-Ct}) \text{ mean} \times \text{probe slope}]}{(28\text{S slope} \times \text{DF})}$$

The slope in the formula was m, which was from equation of line (y = mx + c) of standard curve. Following this, the fold changes of the corrected data were calculated by:

$$\text{Fold changes} = 2^{(\text{Corrected sample-corrected control})}$$

The corrected control was the sample that was designated as the "calibrator" (or 1 × sample), and all other samples were compared to this calibrator sample. Notably, the absolute values calculated for the experimental samples do not have biological meaning, while the relative difference in mRNA abundance between samples were accurate.

2.2.8 Chemotaxis assay

The *in vitro* migration of BMDC in response to CC chemokines was assessed in a 24-well transwell cell culture chamber with 5.0 μm pore size polycarbonate filters. Approximately 2×10^5 cells in 100 μl of IMDM/10 tissue culture media were added to the upper compartment of the chamber. CCL19 (0.3 μM) or CCL20 (100 ng/ml) in 600 μl of IMDM/10 was loaded in the lower compartment. The transwell plate was incubated at 37°C, 5%CO_2 for 4 hours. The cells that had moved through the

polycarbonate filters into the lower compartment were collected and stained with anti-CD11c and anti-I-A/I-E antibodies. The double positive cells (defined as the DC) were counted by flow cytometry and the percentage of migrated BMDC determined by the number of migrated DC divided by the number of DC seeded in the upper compartment. The lower compartment of control chambers contained media alone. The assay was performed in duplicate.

For the desensitisation experiments, BMDC (3×10^6 cells/ml) were incubated for 1 hour at 37°C with 5~10 μg/ml CCL19, control cells were incubated with PBS for 1 hour at 37°C. After washing 3 times with PBS, the cells were resuspended in IMDM/10 media and the number of cells were adjusted. Cells (2×10^5/well) were seeded into the upper compartment of the transwell plate, and the following steps were the same as for the chemotaxis assay described as above.

2.2.9 Labelling cells with the Cell Tracker dyes

To investigate the *in vivo* migration of BMDC and BMMac, cells need to be labelled with fluorescent cell trackers. Vybrant™ CFDA SE Cell Tracker kit (containing CFDA SE (carboxy-fluorescein diacetate, succinimidyl ester; often called CFSE) and CellTracker Orange CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine) were used. The cell tracker dyes contain a chloromethyl group that reacts with thiol, in a glutathione S-transferase-mediated reaction, which is ubiquitous in most cells. Following the reaction, the reagent is transformed into a cell-impermeant fluorescent dye—thioether adduct that can be fixed with a

aldehyde fixatives and detected by FACS flow cytometry. CFSE can be used not only to label the cells but also used to measure the rate of T cell proliferation *in vivo* and *in vitro* due to the fact that its fluorescence is split and inherited by daughter cells after cell division.

The stock of the cell tracker was prepared to make 5 mM final concentration in PBS/0.1%BSA, which was then aliquoted into 5 μl samples and stored at -20°C in the dark until further use. The cells requiring labelling were harvested and adjusted to 10^7 cells/ml in IMDM/10 media. The stock of cell tracker was diluted 1:1000 and added into the cell suspension (final concentration 5 μM) and incubated for 10 minutes at 37°C. After incubation, labelled cells were washed twice with 50 ml of pre-warmed PBS/5%FCS to stop the reaction. The cell suspension was filtered through 40 μm filter to remove any cell debris and then pelleted by centrifugation. The labelled cells were counted and resuspended to the appropriate dilution and used for further experiments.

2.2.10 Analysis of T cell response

2.2.10.1 Isolation of CD4 or CD8 Subsets from leukocytes

In order to analyse the proliferation of CD4 or CD8 T cells in response to BMDC stimulated with recombinant *S. typhimurium*, T cell subsets were isolated (by negative selection) from lymphoid tissues using CD4+ or CD8+ T cell isolation kits following the manufacturer's protocol. Briefly, spleen or lymph nodes were mashed

with a syringe plunger to obtain a single cell suspension in buffer (0.5% BSA (w/v in Milli-Q® water), 2 mM EDTA in PBS, pH 7.2, filter before use and keep cold). After 10 minutes centrifugation at 300 × g, cells were filtered through 40 μm strainer and cell number was counted. A cell suspension at a density of 10^7/40 μl was prepared and 10 μl of biotin antibody cocktail containing biotin-conjugated monoclonal antibodies against CD8a (for CD4 isolation, or CD4 for CD8 isolation), CD11b, CD45R, DX5 and Ter-119 was added to 10^7 cells. After 10 minutes incubation at 4~8°C, 30 μl of buffer was added to the cells followed by addition of 20 μl of anti-biotin MicroBeads per 10^7 total cells. Following another 15 minutes incubation at 4-8°C, the cells were washed with T cell isolation buffer and centrifuged at 300 × g for 10 minutes. Cells were then resuspended at a density of 10^8 total cells per 500 μl of buffer. A MACS column and a MACS separator were chosen depending on the number of cells being magnetically labelled. A LS column (up to 10^8 labelled cells) or MS column (up to 10^7) was placed in the magnetic field of the MACS separator. The column was pre-wetted with 2 ml of buffer and the cell suspension was passed through the column followed by washing three times with 1 ml buffer and the entire eluate was collected, representing the enriched CD4+ (or CD8+) T cell fraction. Purified T cell subsets were counted and used in the further experiments.

2.2.10.2 IFN-γ Enzyme-Linked Immunosorbent Spot (ELISpot) assay

An IFN-γ ELISpot assay was used to evaluate T-cell responses *in vitro*. A 96-well plate was pre-wetted with 70% methanol (v/v in Milli-Q® water) for 1 minute and

washed three times with PBS. The wells were then coated overnight at 4°C with an IFN-γ capture antibody (final concentration 10 μg/ml) in sterile PBS. On the following day, the plate was washed with PBS and blocked with T-cell media (RPMI 1640, 10% FCS, 50 μM β-ME, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate) for 2 hours at 37°C. Responder cells (5×10^4~2×10^5 of CTL cells or fresh T cells from GRb mice) in 50 μl of T-cell media were added to each well and the plate was incubated at 37°C, 5% CO_2 for 1 hour to allow the cells to settle down. For *in vitro* analysis of CTL lines, 10^3~10^5 stimulators, such as BMMac or BMDC, either pulsed with 1 μM peptide or infected with recombinant *Salmonella*, were added to each well in 50 μl of T-cell media containing gentamicin (final concentration 50 μg/ml in total volume). For *ex vivo* T cell responses, media containing 1 μM $NP_{383-391}$ peptide in 50 μl was added. After 24 hours of incubation at 37°C, 5% CO_2, wells were washed six times with the 150 μl/well PBS/0.01% Tween®20. The wells were then incubated for 2 hours at 37°C with a biotinylated anti-IFN-γ antibody in 0.5% BSA in PBSa (w/v, final concentration 2 μg/ml). After washing six times with PBS/0.01% Tween®20, 100 μl of 1:1000 diluted Streptavidin-HRP™ enzyme conjugate in sterile PBS was added and the plate was incubated at room temperature for 1 hour. After washing three times with PBS/0.01% Tween®20 followed by three times with PBSa, spots were developed by incubation for 4 minutes with 3-amino-9-ethylcarbazole (AEC) (dissolved 0.4 mg/ml in dimethylformamide) in 0.1 M acetate buffer (0.0074 N acetic acid, 0.018 M sodium acetic, pH 5.1), 0.015% (v/v) hydrogen peroxide was added to the solution before addition. AEC was removed by washing the plate with the running

water. Following this, the plate was then left to dry overnight in the dark, spots formed by IFN-γ-secreting cells were counted under the microscope using the Qwin software (Leica).

2.2.10.3 T cell proliferation assay using [^{3}H] thymidine

T cell proliferation in response to BMDC or BMMac stimulation after *S. typhimurium* infection was evaluated using [^{3}H] thymidine (Amersham). T cells (10^4) in 100 μl of T cell media isolated from GRb mice were seeded into the wells of U-bottom 96-well plates. BMDC or BMMac were infected with recombinant *S. typhimurium* or peptide pulsed cells were used as stimulators. After infection or peptide pulsing, BMDC or BMMac were harvested and 10^3 cells in 100 μl media containing gentamicin (final concentration 50 μg/ml) were added to the wells. The plate was then incubated in a 37°C, 5%CO_2 incubator for 4 days. On day 4, the cells were pulsed with [^{3}H] thymidine (0.5 μCi/well in 20 μl) for 16 hours. Following this, the cells were washed and passed through a glass fibre filter. The filter was dried and sealed with a plastic bag containing 5 ml of betaplate scint, which was then loaded onto the 1450 Microbeta Trilux Scintillation Counter, and the incorporation of thymidine into the synthesised cellular DNA was determined.

2.2.10.4 T cell proliferation assay using CFSE labelling

To evaluate the capacity of BMDC or BMMac to present intracellular bacteria-derived antigens to T cells, proliferation of T cells upon stimulation was analysed by CFSE labelling. T cells were isolated from lymph nodes of GRb mice; when

purified T cell subsets were used (CD4 or CD8), T cells were purified by negative selection using MACS T cell isolation kit as described in section 2.2.10.1. Then, 10^7 cells/ml were labelled with 5 μM CFSE as described in 2.2.9. After labelling, 10^5 T cells in 100 μl were added to the wells of U-bottom 96-well plates. After infection with recombinant *S. typhimurium* or peptide pulsing, 10^4 BMDC or BMMac in 100 μl in the presence of 50 μg/ml gentamicin were also added to the wells. The culture was incubated at 37°C, 5%CO_2 for 4 days, the cells were harvested and analysed by flow cytometry, and the proliferation of T cells was measured by the dilution of CFSE-fluorescence intensity at various time points.

2.2.11 Adoptive transfer

2.2.11.1 Adoptive transfer of BMMac or BMDC

The *in vivo* migration of BMDC or BMMac after *S. typhimurium* infection was investigated by the adoptive transfer of CFSE or CMTMR labelled cells. BMMac or BMDC were prepared as previously described in 2.2.2.3 or 2.2.2.5, and infection was performed as described in section 2.2.3.3. Overnight incubation in the presence of 50 μg/ml gentamicin was used to eliminate the extracellular bacteria. The cells were harvested and labelled with CFSE or CMTMR as described in section 2.2.9. Following this, CFSE labelled cells were resuspended at a density of 1~2 × 10^7/ml, and 500 μl of the cell suspension was i.p. injected into a HLA-B27 transgenic mouse, 3 mice per group. The mice were observed daily for 2 days. On day 2, all the mice were sacrificed by a rising concentration of CO_2, and tissues

taken to make single cell suspensions for analysis. First of all, 5 ml of PBS was injected into the peritoneal cavity by a 5 ml syringe and 25 G needle, and the abdomen was massaged for two minutes. The peritoneal fluid was then aspirated into the syringe and the recovered lavage was expelled into a 5 ml tube on ice for analysis. Following this, spleen, liver, Peyer's Patches (PPs) mesenteric lymph nodes (LN), inguinal LN and popliteal LN were isolated and stored in PBS. To analyse the number of migrating CFSE labelled cells in different organs, single cell suspensions were prepared from each organ. Spleen and liver were cut into pieces and digested with tissue digesting media (1 mg/ml collagenase type IV and 0.5 mg/ml DNase I in RPMI/5%FCS) for 30 minutes at 37°C with constant shaking. All the LNs and PPs were mashed with the edge of two frosted slides. Cells were then passed through a 40 μm strainer, washed in FACS wash buffer (0.1% FCS, 0.01% sodium azide in PBS) and resuspended into 1% (w/v) PFA. All samples were then analysed by flow cytometry with the 2×10^6 cells collected for each sample. CFSE positive cells that appeared on FL1 channel were calculated. The mice with no transfer of CFSE labelled cells were used as controls.

2.2.11.2 Co-transfer of GRb T cells and BMDC

In order to assess the capacity of the BMDC to present bacterial antigens to antigen specific naïve T cells *in vivo*, GRb T cells and recombinant *S. typhimurium* infected BMDC were co-transferred into HLA-B27 mice. Firstly, lymphatic T cells were isolated from two GRb × HLA-B27/β_2m mice; cells were pooled and labelled with CFSE as described in section 2.2.9. One million CFSE labelled GRb T cells in

200 μl of PBS were then intravenously (i.v.) injected into HLA-B27 mice via the tail vein. On the following day, recombinant *S. typhimurium* infected or peptide pulsed BMDC were harvested and 5×10^6 cells in 500 μl of PBS were i.p. injected into HLA-B27 mice previously injected with GRb T cells, 3 mice each group. Mice were monitored daily. Up to 8 days after BMDC transfer, spleen and lymph nodes were taken for analysis. Cells were isolated as described in section 2.2.11.1. After washing with FACS wash buffer, 2×10^6 cells from each organ were aliquoted into the wells of U-bottom 96-well plates, extracellular FACS staining was performed as described in section 2.2.4.1 and intracellular cytokine staining was carried out as described in section 2.2.4.2. TCR Vβ7 antibody staining was used, since >90% of lymphatic T cells from GRB × HLA-B27/hβ_2m mice expressed the transgene-derived human Vβ7 molecule (Roddis et al., 2004), allowing GRb lymphatic T cells to be visualised by this antibody staining. The stained cells were then analysed by flow cytometry and T cell proliferation was assessed by the loss of intensity of the CFSE fluorescence. The function of T cells was analysed by intracellular staining of IFN-γ or granzyme B.

2.2.12 Statistical analysis

Data were analysed using S-Plus (r) 6.2 for Windows and Microsoft Excel 2002. Statistical analysis was performed by Xikun Wu from Institute for Animal Health according to the directions given by the Institute for Animal Health statistics advisory desk. In chapter 4, statistical significance was analysed using one-way

ANOVA (Analysis of Variance), and two-sample student's T-test was used in chapter 5.

Chapter 3: *In vitro* optimisation of experimental system

3.1 Introduction

In this study, an adoptive transfer was designed to study trafficking and antigen transportation by macrophages and DC. Since tissue derived macrophages and DC can only be isolated in small numbers, BMMac or BMDC were used in this study in order to do large-scale adoptive transfer. Experimentally, macrophages and DC can be propagated from bone marrow precursors cultured with distinct cytokines. Macrophages are generated in response to M-CSF (also called CSF-1) and DC are produced in response to GM-CSF (Caux et al., 1997; Pannetier et al., 2004; Szabolcs et al., 1996).

Salmonella is a known causative agent of ReA and therefore was used as the infection agent and source of antigen in this experimental system. As described in the materials and methods, various attenuated strains are available in our laboratory. As *S. typhimurium* can be cytotoxic for cultured macrophages by inducing apoptosis (Chen et al., 1996a; Monack et al., 1996), and healthy live cells were required for adoptive transfer, BMMac were used to determine the bacteria strain, infectious dose and time point at which infected cells were harvested. BMMac and BMDC were then infected with *Salmonella* under these predetermined conditions and the consequences of infection subsequently compared by assessing infection rate, changes of cell surface molecules, NO production and cytokine secretion.

The aim of the following studies was to optimise the *in vitro* experimental system to obtain viable *S. typhimurium* infected macrophages and DC carrying *Salmonella* intracellularly. These infected cells will be then used for adoptive transfer into mice and their migratory properties assessed.

3.2 Results

3.2.1 Generation of primary cultures of BMMac and BMDC

3.2.1.1 Generation of BMMac

BMMac were generated by culturing freshly isolated bone marrow from HLA-B27 mice in IMDM medium supplemented with 20% Ladmac cell culture supernatant as described in the materials and methods, which is consistent with the reported use of the supernatant from this cell line (Ponniah et al., 1996). To standardise the BMMac culture, supernatants were collected from different batches of Ladmac cell culture over 10 months to obtain sufficient amounts of supernatant for future BMMac culture. To assure that these supernatants contained comparable amounts of M-CSF, an ELISA was used to quantify the M-CSF present. From a standard curve generated by serial dilution of mouse recombinant M-CSF (Figure 3.1 A), the amount of M-CSF was calculated and compared among different batches of culture supernatant (Figure 3.1 B). It was found that these supernatants contained levels of M-CSF ranging from 30~45 ng/ml. Therefore, the final concentration of M-CSF used in BMMac culture was equivalent to 6~9 ng/ml recombinant M-CSF.

3.2.1.2 Generation of BMDC

3.2.1.2.1 Selection of FCS

Our protocol to generate BMDC with GM-CSF was adapted from previous publications (Lutz et al., 1999; Lutz et al., 2000). In these studies, the batch of FCS influenced both cell yield and DC maturation (Lutz et al., 1999). A major component in FCS that may potentially influence the maturation state and

outgrowth of DC is endotoxin contamination. Thus, 8 batches of FCS (Table 3.1) were obtained from different companies and tested for endotoxin levels by LAL assay (Ochiai et al., 2003; Takagi et al., 1981). The amount of endotoxin contamination in these samples was calculated according to a standard curve generated from serial dilution of commercial endotoxin with a known concentration in the LAL assay. Overall, comparing 8 samples as shown in Figure 3.2 A, we found that sample H contained the highest concentration of endotoxin (nearly 50 EU/ml). In contrast, the level of endotoxin in the other 7 samples was less than 5 EU/ml. Among these 7 FCS samples (Figure 3.2 B), samples A, D, E contained the lowest level of endotoxin (1 EU/ml). As sample E had limited availability from the suppliers, sample A and D were chosen and tested for effects in DC culture, with samples C and H used as intermediate and high endotoxin controls respectively.

DC were generated from bone marrow culture in the presence of GM-CSF in medium supplemented with 10% of samples A, C, D or H. DC were harvested at day 7, and the maturation state and contamination with granulocytes analysed by flow cytometry as described in materials and methods. CD11c was used as a dendritic cell marker throughout this study. Gr-1 was used as a marker indicating contamination of granulocytes. I-A/I-E and other costimulatory molecules such as CD40, CD54, CD80 and CD86 were used to measure the maturation of DC. Figure 3.3 shows that cells generated from a culture supplemented with FCS sample A had the lowest expression of Gr-1 (7.7%), I-A/I-E (56.1%), CD54 (68.9%), CD86 (34.6%) and CD80 (76.7%) and the second lowest expression of CD40 (29.5%). Notably, cells grown in sample H,

which contained the highest level of endotoxin, had the highest Gr-1 expression (13.6%) indicating the greater higher contamination with granulocytes. Therefore, sample A was chosen as the source of FCS for future BMDC culture throughout this study.

3.2.1.2.2 Growth state of BMDC

To further optimise production of BMDC, we analysed how varying the number of seeded bone marrow cells and the duration of culture affected DC yield, purity and maturation state. Bone marrow cells were serially diluted from 5×10^6 to 1.5×10^5, and seeded into individual wells of 6-well plates followed by culture with recombinant GM-CSF. At day 5 and day 7, nonadherent and adherent cells were harvested separately. Cell counting was performed and cell yield including both populations was calculated as the number of total cells harvested from the culture divided by the number of originally seeded cells. After evaluation of cell yield, the cells were then double stained with antibodies against CD11c and I-A/I-E or Gr-1. Upon analysis of Gr-1 expression, we found that the nonadherent population was contaminated with 15% ~ 40% of granulocytes, in comparison with only 8% ~ 12% granulocyte contamination of the adherent population (Figure 3.4). In terms of I-A/I-E expression, we found that there were two populations generated from our DC culture regardless of number of seeded cells: cells with high levels of I-A/I-E expression and cells with low levels of I-A/I-E (Figure 3.4). In order to achieve a greater number of adherent cells, 100 mm bacteriological petri dishes were replaced with T75 tissue culture flasks.

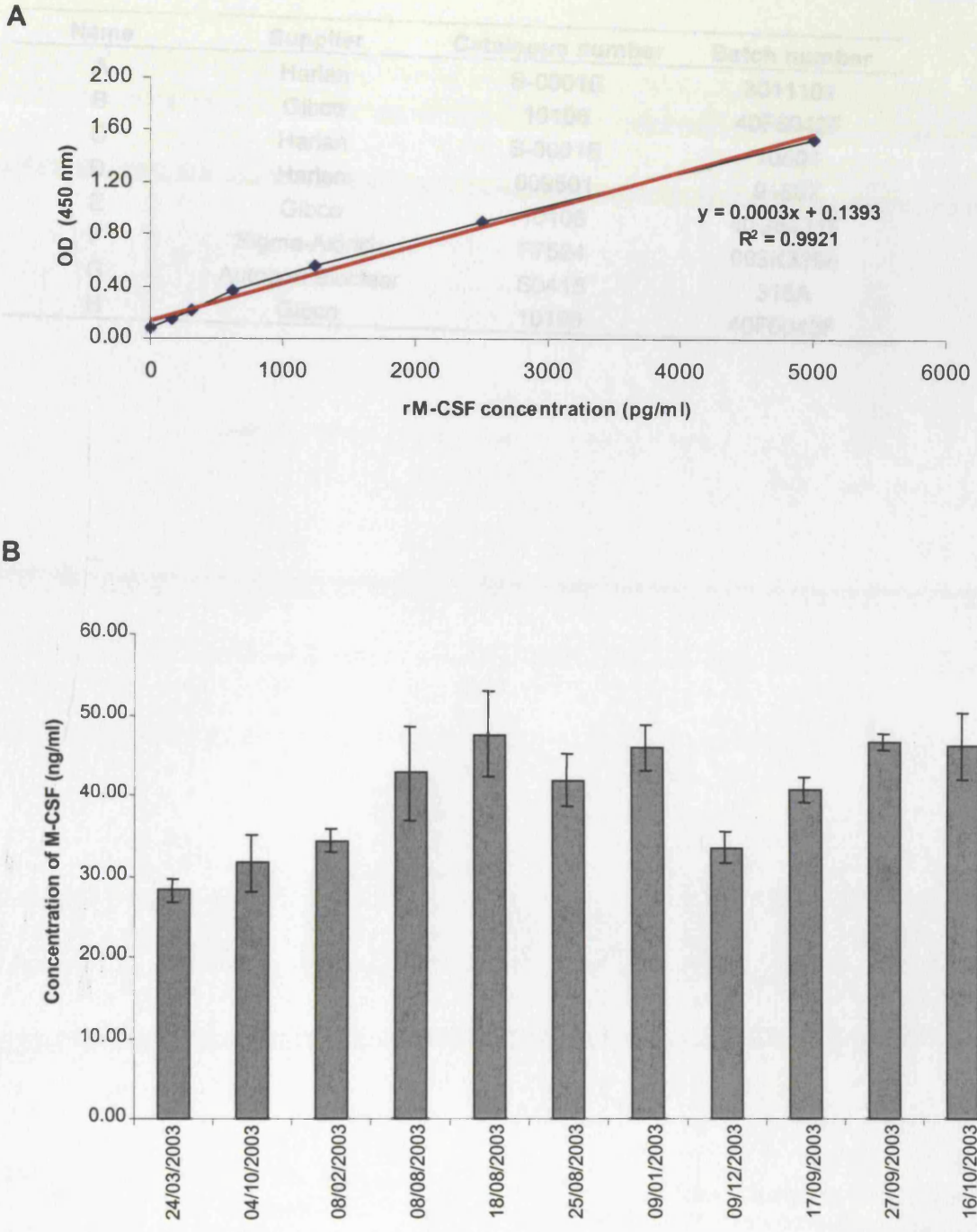


Figure 3.1 Quantification of the M-CSF contained in the supernatant harvested from different batches of Ladmac cell culture. Ladmac cells were cultured in DMEM/10 medium for two weeks, the supernatant was harvested and the level of M-CSF contained in the supernatant was quantified by ELISA using murine recombinant M-CSF to generate a standard curve (A). The equivalent concentration of M-CSF in different batches of culture supernatant (B) was calculated according to the equation generated from the standard curve. Each bar indicates different batch of Ladmac cell culture supernatant. Results represent the mean values ± SD of triplicates for each sample in the assay.

Table 3.1 Source of the FCS tested in LAL assay.

Name	Supplier	Catalogue number	Batch number
A	Harlan	S-0001E	3011101
B	Gibco	10106	40F6042F
C	Harlan	S-0001E	10504
D	Harlan	009501	91607
E	Gibco	10106	40G8431F
F	Sigma-Aldrich	F7524	093K3396
G	Autogen Bioclear	S0415	318A
H	Gibco	10106	40F6043F

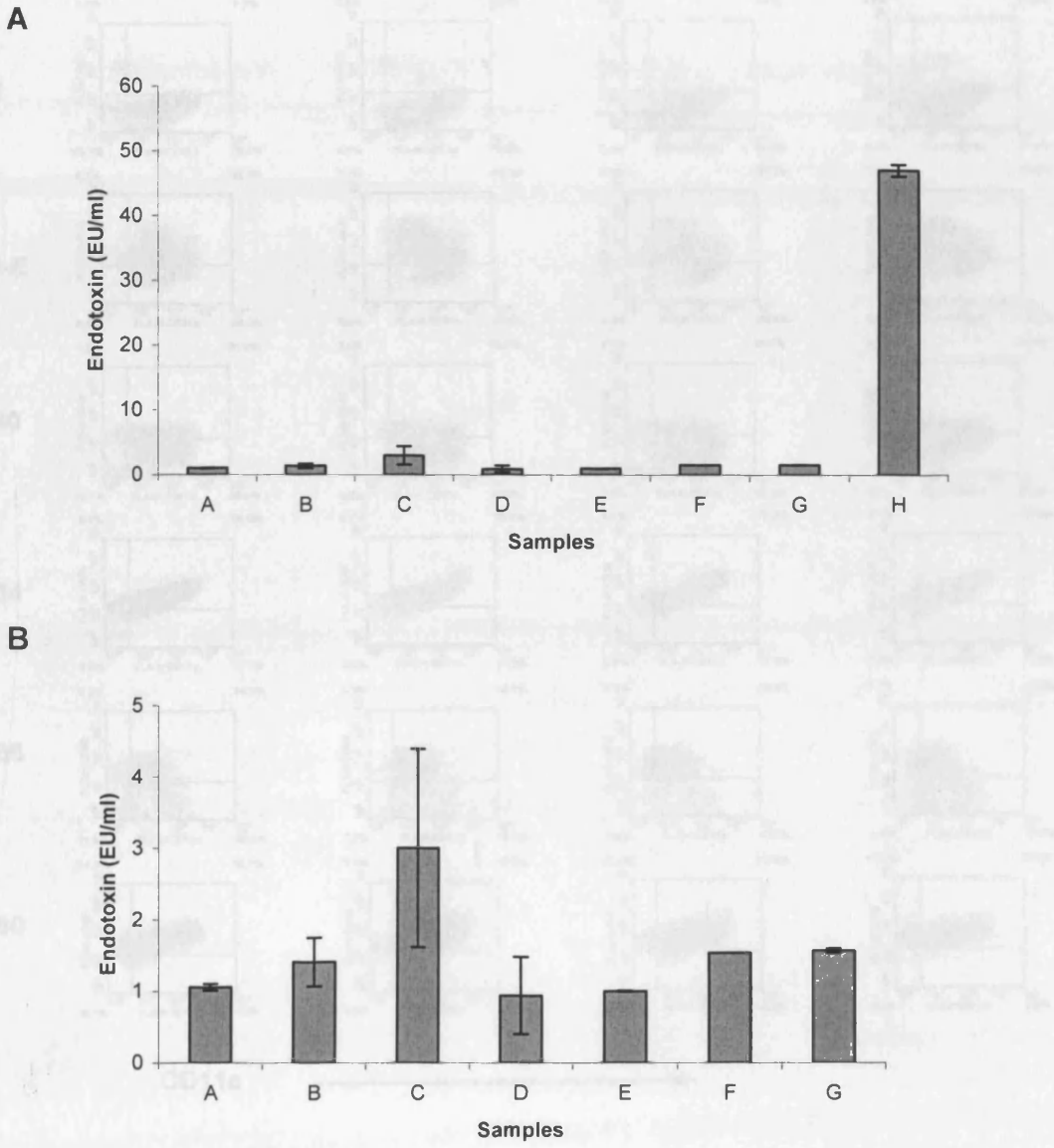


Figure 3.2 Comparison of the endotoxin level contained in different batches of FCS using the LAL assay. Eight samples of FCS obtained from different companies were tested, and the endotoxin level of samples was calculated according to the standard curve. (A) Overall comparison of 8 samples. (B) Comparison of 7 samples excluding H which contains a higher level of endotoxin compared to the other 7 samples. Results represent mean values ± SD of triplicates for each sample.

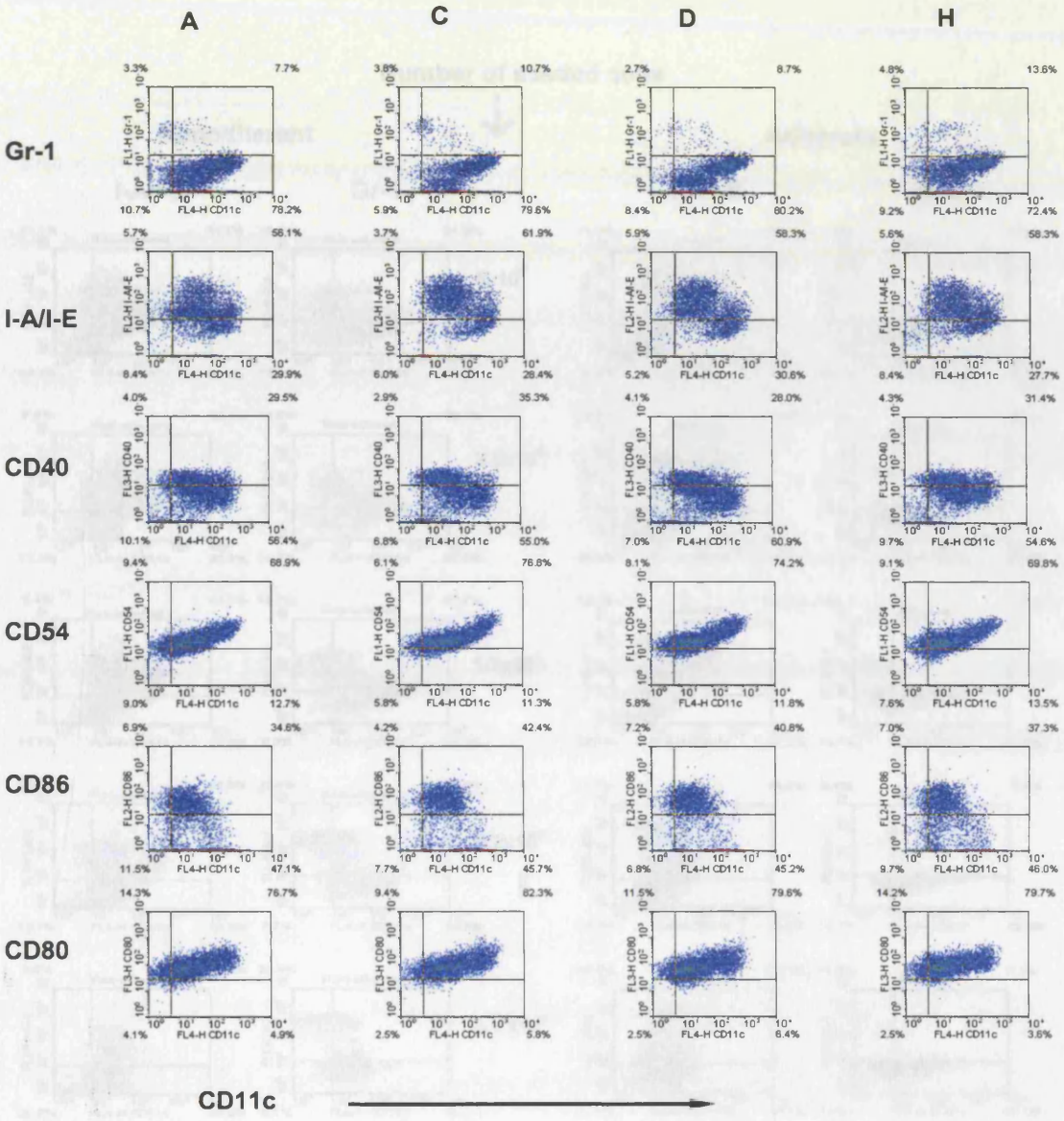


Figure 3.3 Further selection of low endotoxin FCS based on effects in DC culture. Four samples of FCS (A, C, D and H) chosen from the LAL assay were used in 7 day DC cultures in the presence of GM-CSF. DC were harvested at day 7 and the maturation state of the DC was analysed by flow cytometry. Cells were stained with CD11c (APC), Gr-1 (FITC), I-A/I-E (PE), CD40 (Biotin), CD54 (FITC), CD80 (Biotin) and CD86 (PE). Biotinylated antibodies were followed by staining with streptavidin-Cy-chrome. The expression of the other cell surface markers were analysed on CD11c (+) cells. The percentage of cells falling into each quadrant is indicated.

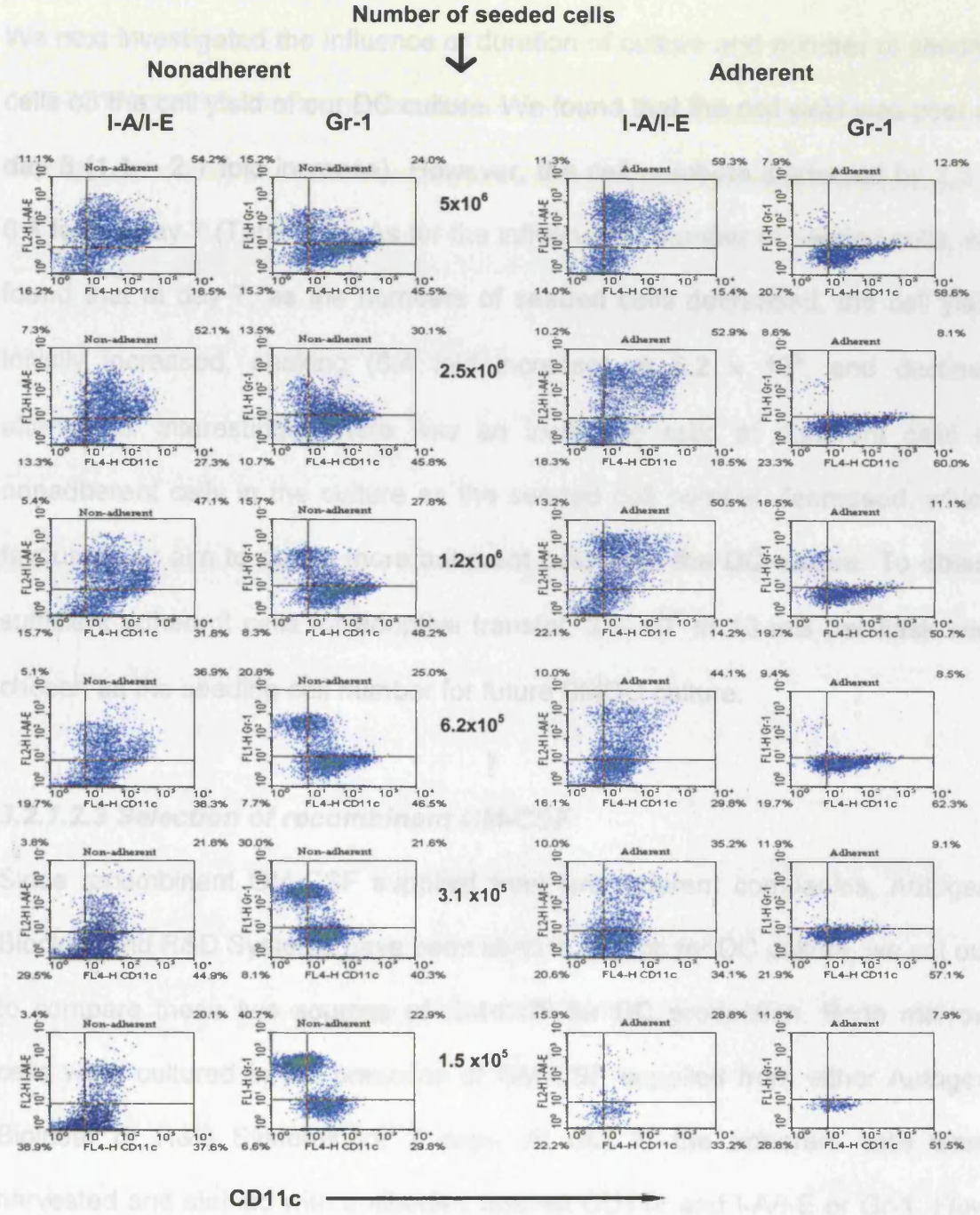


Figure 3.4 Comparison of cell surface marker expression of non-adherent and adherent cells generated from DC cultures seeded with different numbers of bone marrow cells. Bone marrow cells were serially diluted and seeded into 6-well plates followed by culture with recombinant GM-CSF for 7 days. At day 7, nonadherent and adherent cells were harvested separately. The cells were stained with antibodies against CD11c (APC conjugate), I-A/I-E (PE) and Gr-1 (FITC). The expression level of I-A/I-E and Gr-1 of CD11c (+) cells were compared (percentage shown on the top right corner).

We next investigated the influence of duration of culture and number of seeded cells on the cell yield of our DC culture. We found that the cell yield was poor at day 5 (1.1 ~ 2.1 fold increase). However, the cell numbers increased by 2.3 ~ 6.4 fold at day 7 (Table 3.2). As for the influence of number of seeded cells, we found that at day 7, as the numbers of seeded cells decreased, the cell yield initially increased, peaking (6.4 fold increase) at 6.2×10^5, and declined afterwards. Interestingly, there was an increased ratio of adherent cells to nonadherent cells in the culture as the seeded cell number decreased, which favoured our aim to obtain more adherent cells from the DC culture. To obtain sufficient adherent cells for adoptive transfer, 2×10^6 in 10 mls per flask was chosen as the seeding cell number for future BMDC culture.

3.2.1.2.3 Selection of recombinant GM-CSF

Since recombinant GM-CSF supplied from two different companies, Autogen Bioclear and R&D Systems have been used in our lab for DC culture, we set out to compare these two sources of GM-CSF for DC production. Bone marrow cells were cultured in the presence of GM-CSF supplied from either Autogen Biolcear or R&D Systems for 7 days. At day 7, the adherent cells were harvested and stained with antibodies against CD11c and I-A/I-E or Gr-1. Flow cytometry analysis showed that cells generated from the two different sources of GM-CSF had comparable expression of CD11c and I-A/I-E, but slightly lower expression of Gr-1 was observed on cells supplemented with GM-CSF from R&D Systems (Figure 3.5 A), indicating lower contamination with granulocytes. In addition, there was about 3-fold increase in adherent cells in the cultures with

GM-CSF from R&D Systems, compared to only a 1-fold increase in cultures supplemented with the GM-CSF from Autogen Bioclear (Figure 3.5 B), thus, GM-CSF from R&D systems was selected.

3.2.2 Characterisation of BMMac and BMDC

3.2.2.1 Phenotypic analysis of BMMac

Macrophages were generated by culturing bone marrow cells isolated from HLA-B27 transgenic mice for 3 weeks in medium supplemented with 20% Ladmac cell supernatant. Adherent cells were harvested and stained with a panel of antibodies against various cell surface molecules as described in materials and methods. Flow cytometric analysis showed that BMMac displayed expression of the myeloid cell related markers CD11b and F4/80, but not CD11c. Cells did not express MHC class II (I-A/I-E), whereas class I H-2k^d and HLA-B27 expression was detected (Figure 3.6). BMMac lacked expression of Gr-1, CD3 and B220 (Figure 3.6), thus indicating an absence of granulocytes, B cells and T cells in our BMMac culture.

3.2.2.2 Phenotypic analysis of BMDC

BMDC were generated from HLA-B27 transgenic bone marrow cells by culture for 7 days from HLA-B27 mice in the presence of GM-CSF and stained with the same panel of antibodies as BMMac. From flow cytometry analysis (Figure 3.7) we found that our BMDC displayed high expression of CD11b and CD11c, and low expression of F4/80. BMDC expressed similar levels of class I H-2Kd and HLA-B27. Class II I-A/I-E expression showed subdivision into I-A/I-Ehigh and

Table 3.2 Selection of the seeded cell numbers and harvest day for DC culture. Since the number of seeded cells and the day of harvest could affect the maturation state and yield of cultured DC, these two conditions were optimised. Serial dilutions of BM cells were seeded in 6-well plates and incubated in the presence of GM-CSF for 7 days. At day 5 and 7, non-adherent and adherent cells were harvested and counted separately. The yield of culture was indicated by the fold change of cell numbers, which was calculated as number of total cells harvested from the culture divided by the number of originally seeded cells.

		Day 5				Day7		
No. of seeded cells	**State**	**Cell no.**	**Overall**	**Yield**	**State**	**Cell no.**	**Overall**	**Yield**
5.0×10^6	N*	5.2×10^6	6×10^6	1.2	N	1.1×10^7	1.4×10^7	2.8
	A**	8 x 105			A	3.3×10^6		
2.5×10^6	N	3.2×10^6	4×10^6	1.6	N	6.6×10^6	9.1×10^6	3.6
	A	8×10^5			A	2.5×10^6		
1.2×10^6	N	2×10^6	2.6×10^6	2.1	N	3×10^6	4.9×10^6	3.9
	A	6.4×10^4			A	1.9×10^6		
6.2×10^5	N	6.4×10^5	6.9×10^6	1.1	N	1.5×10^6	4×10^6	6.4
	A	4.8×10^4			A	2.5×10^6		
3.1×10^5	N	3×10^5	3.4×10^5	1.1	N	4.3×10^5	1.4×10^6	4.8
	A	4×10^4			A	1×10^6		
1.5×10^5	N	1.8×10^5	1.9×10^5	1.3	N	1.8×10^5	3.4×10^5	2.3
	A	1.6×10^4			A	1.6×10^5		

* N: Non-adherent ** A: Adherent

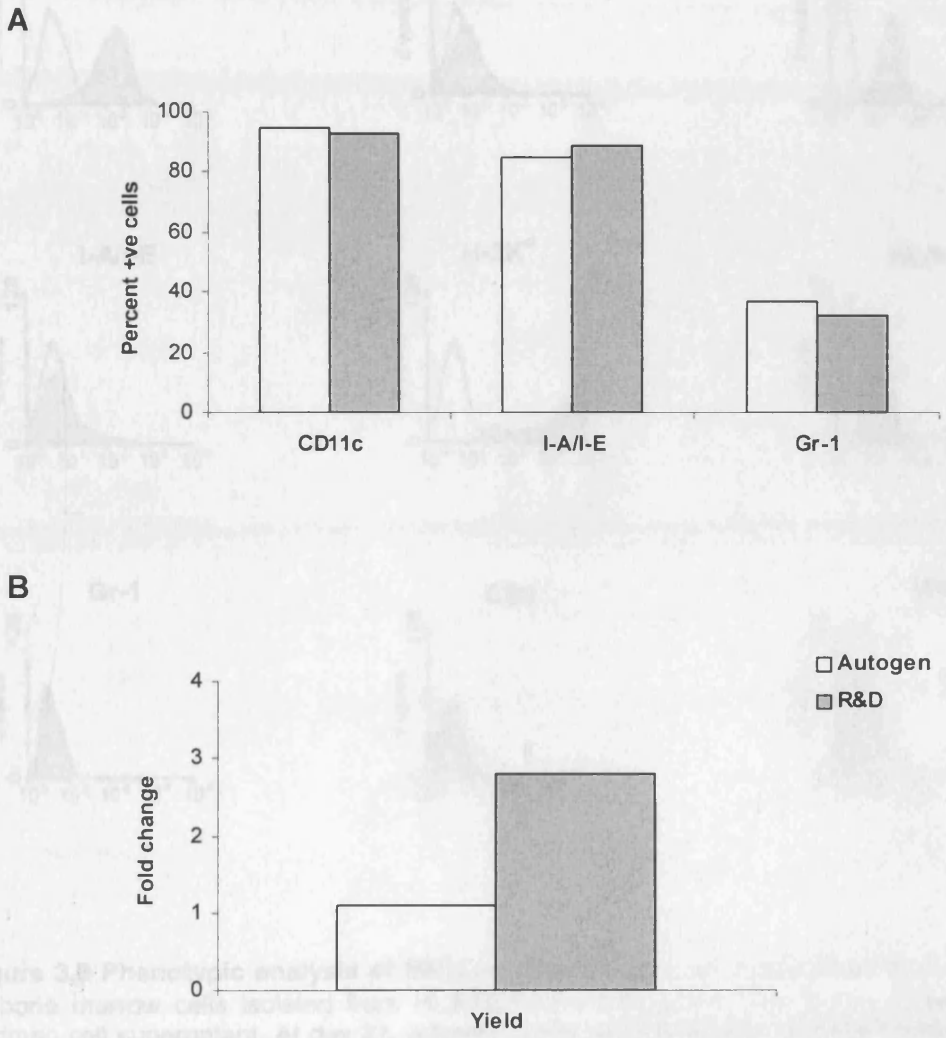


Figure 3.5 Comparison of the effect of recombinant murine GM-CSF supplied by Autogen Bioclear and R&D Systems on DC culture. BMDC were generated in the presence of GM-CSF. At day 7, the adherent cells were harvested and stained with the antibodies against CD11c (APC conjugate), I-A/I-E (PE) and Gr-1 (FITC). (A) Percentage of cells expressing indicated markers. (B) The yield of culture expressed as fold change, which was calculated as the number of harvested cells divided by the number of seeded bone marrow cells.

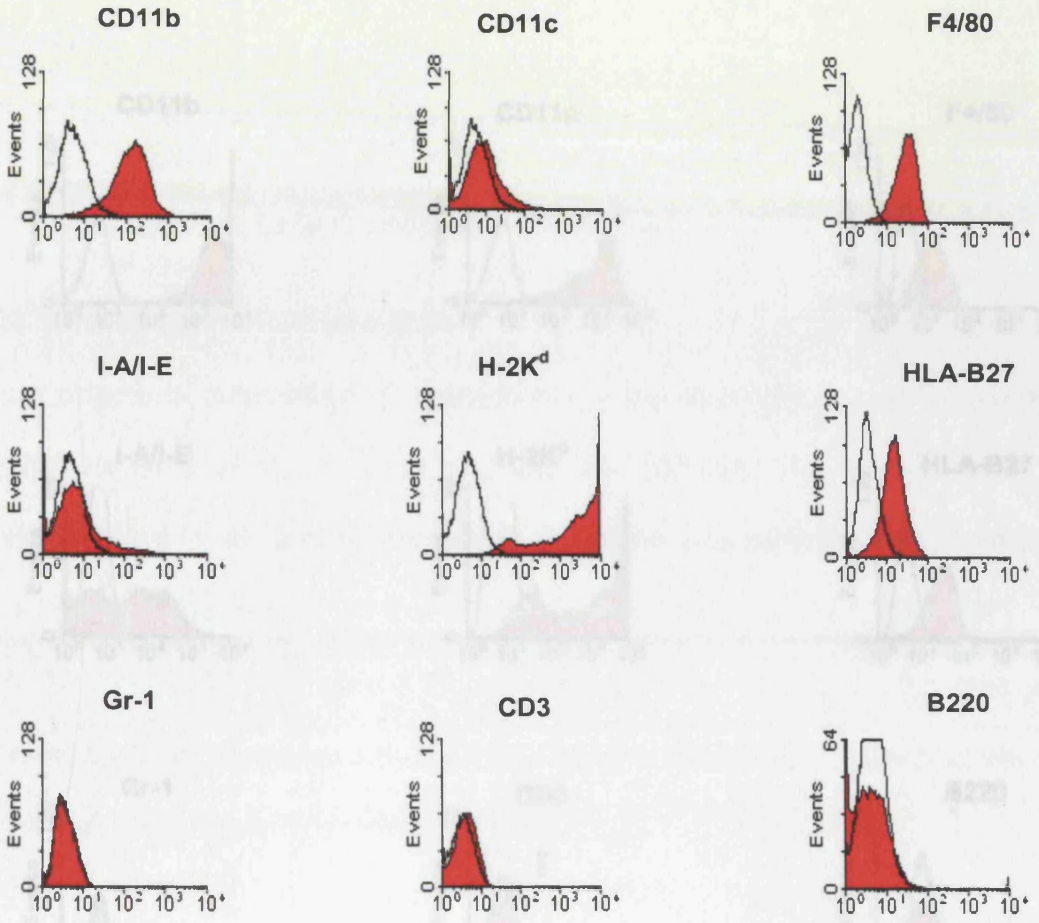


Figure 3.6 Phenotypic analysis of BMMac. Macrophages were generated by 3-week culture of bone marrow cells isolated from HLA-B27/β2m transgenic mice in the presence of 20% Ladmac cell supernatant. At day 21, adherent cells were harvested and the phenotype of cells analysed by flow cytometry. Cells were stained with a panel of antibodies against the following cell surface molecules: CD11b (Biotin), CD11c (Biotin), F4/80 (FITC), I-A/I-E (PE), H-2Kd (PE), HLA-B27 (FITC), Gr-1 (FITC), CD3 (APC) and B220 (PE). Biotinylated antibodies were detected with streptavidin-Cy-chrome. Each plot shows expression of the indicated cell surface marker as a solid red histogram. Background staining using isotype-matched control antibodies is shown as an empty histogram. These data are representative of all BMMac preparations used throughout the studies.

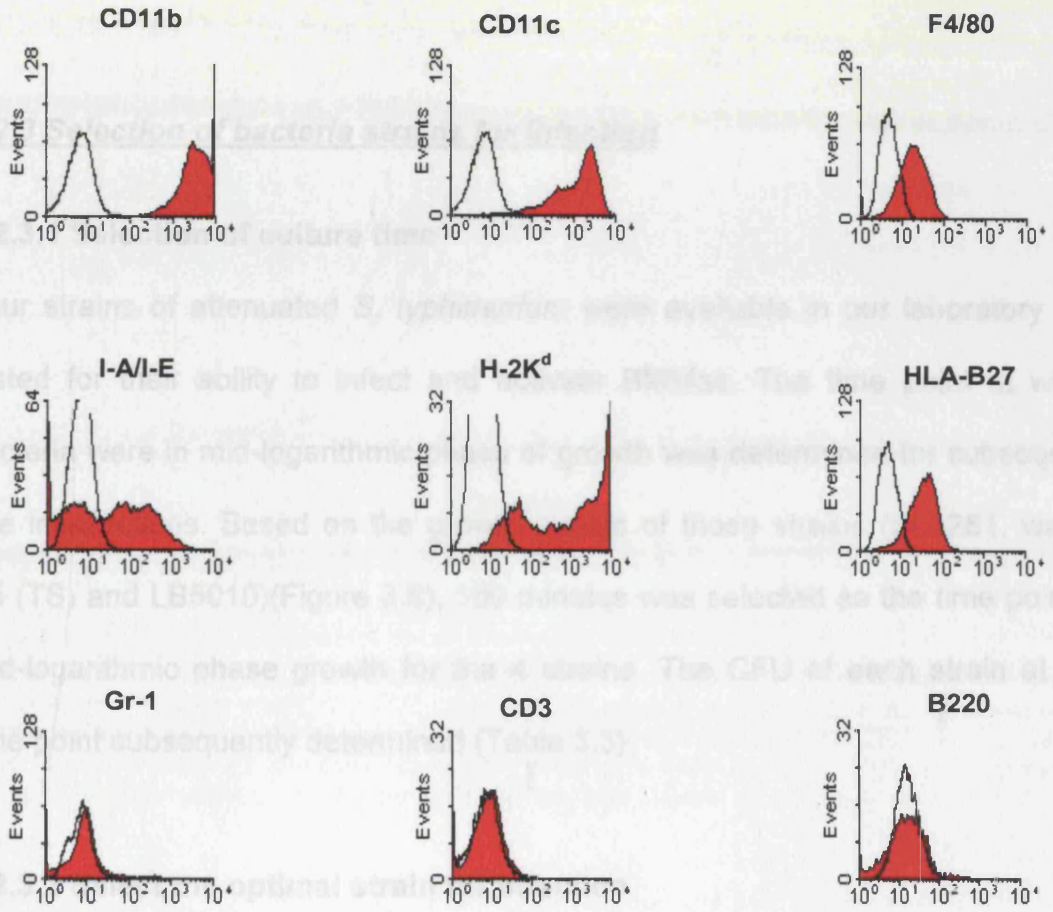


Figure 3.7 Phenotypic analysis of BMDC. DC were generated by 7-day culture of bone marrow cells isolated from HLA-B27/β2m transgenic mice in the presence of the GM-CSF supplied by R&D Systems. At day 7, adherent cells were harvested and the phenotype of cells analysed by antibody staining and flow cytometry. Cells were stained with a panel of antibodies against: CD11b (Biotin), CD11c (Biotin), F4/80 (FITC), I-A/I-E (PE), H-2Kd (PE), HLA-B27 (FITC), Gr-1 (FITC), CD3 (APC) and B220 (PE). Biotinylated antibodies were detected with streptavidin-Cy-chrome. Each plot shows expression of the indicated cell surface marker as a solid red histogram. Background staining using isotype-matched control antibodies is shown as an empty histogram. These data are representative of all BMDC preparations used throughout the studies.

I-A/I-Elow populations. As for BMMac, Gr-1, CD3 and B220 expression was not detected.

3.2.3 Selection of bacteria strains for infection

3.2.3.1 Selection of culture time

Four strains of attenuated *S. typhimurium* were available in our laboratory and tested for their ability to infect and activate BMMac. The time point at which bacteria were in mid-logarithmic phase of growth was determined for subsequent use in infections. Based on the growth curves of those strains (SL3261, waaN, C5 (TS) and LB5010)(Figure 3.8), 180 minutes was selected as the time point of mid-logarithmic phase growth for the 4 strains. The CFU of each strain at this time point subsequently determined (Table 3.3).

3.2.3.2 Select the optimal strain for infection

BMMac were infected with SL3261 at M.O.I's of 10, 50 or 100, and the expression of CD40 on infected F4/80 (+) macrophages analysed as a marker of activation. Uninfected and LPS treated cells served as the negative and positive controls respectively. As shown in Figure 3.9 uninfected macrophages expressed low levels of CD40 (4.2%), in contrast with the high expression of CD40 on LPS treated macrophages (95.8%). After infection with SL3261 at 10 M.O.I, 37% of F4/80 (+) cells expressed CD40 compared to 11% and 4.2% at M.O.I's of 50 and 100 respectively.

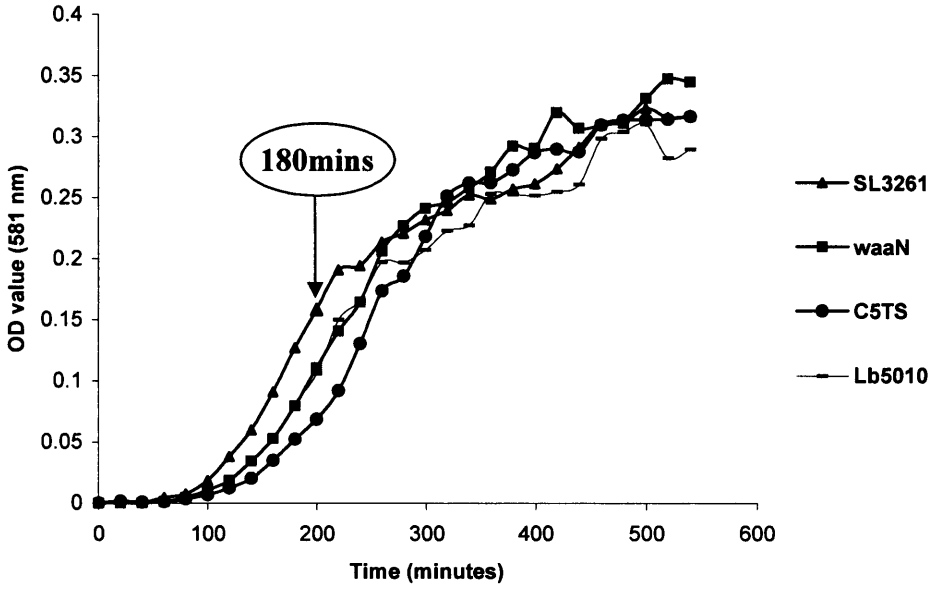


Figure 3.8 Growth curves of 4 attenuated strains of *S. typhimurium*. Stocks of 4 strains of *S. typhimurium* (SL3261, waaN, C5 (TS) and LB5010) were thawed in LB broth. After 16 hours incubation, the bacterial culture was diluted and further incubated, and the OD values at 581nm were recorded every 20 minutes. The optimal time point (180 minutes) of the mid logarithmic phase of bacterial growth, as indicated by the arrow, was chosen for infection.

Table 3.3 The mean CFU count of 4 strains of *S. typhimurium* at 180 minutes. The stocks of 4 strains were thawed in LB broth. After 16 hours incubation, bacterial cultures were diluted and further incubated for 180 minutes. Bacteria were then pelleted and resuspended in a known volume, and then serial dilutions were performed and plated onto LB agar plates. The CFU were counted after overnight incubation. Results represent mean values of three replicate experiments.

Bacteria strain	Mean CFU
SL3261	1.45×10^8 CFU/ml
waaN	8.57×10^7 CFU/ml
C5 (TS)	5.77×10^7 CFU/ml
Lb5010	8.3×10^7 CFU/ml

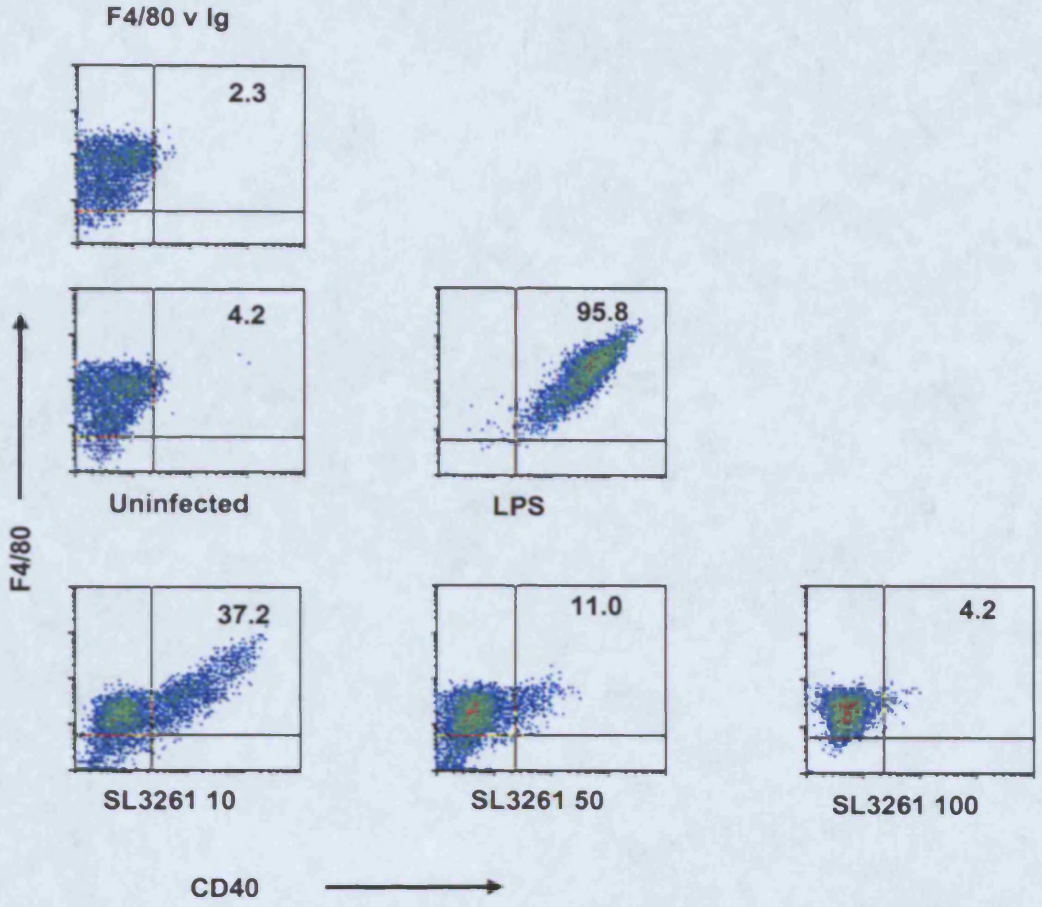


Figure 3.9 Analysis of the effect of SL3261 infection on CD40 expression by BMMac. BMMac were infected with SL3261 at various MOI as indicated. Cells were harvested 24 hours post infection and double stained with antibodies against F4/80 (PE) and CD40 (Cy-chrome). The percentage of double positive staining cells is shown on the top right corner of density plot. The plot on the top row is the control of F4/80 versus isotype control antibody, from which the quadrant was set. Uninfected cells and LPS treated cells serve as controls for the infection. Data are the representative of 3 experiments.

As shown in Figure 3.10, 8.3% of uninfected macrophages expressed CD40, in contrast with 70.1% LPS treated macrophages. After infection with waaN at 10 M.O.I, 51.2% of macrophages expressed CD40 (+); this decreased to 34.7% and 11.1% at 50 and 100 M.O.I respectively.

In contrast to SL3261 and waaN, infection with increasing doses of C5 (TS) did not lead to reductions in CD40 expression. Even when the M.O.I increased to 500, there were still 43.6% of infected cells expressing CD40 (Figure 3.11).

We then set out to compare the activation caused by infection macrophages with each of these 4 strains in the same experiment. Macrophages were infected at 10 M.O.I and levels of CD40 and CD54 analysed. We found (Figure 3.12) that uninfected macrophages did not express CD40 and had very low-level expression of CD54. Macrophages treated with LPS had high expression of CD40 and CD54. In this experiment, infection with SL3261 resulted in a slightly increased expression of CD40 but no increase of CD54 as is also observed upon infection with waaN. Lb5010 infected macrophages showed moderately increased CD40 while CD54 remained at a low level of expression. However, for the C5 (TS) infected macrophages, CD40 and CD54 were both substantially up-regulated compared with uninfected macrophages and the other 3 bacterial infection groups. This indicated that C5 (TS) at 10 M.O.I could effectively activate the BMMac resulting in high expression of cell surface receptors. Therefore, C5 (TS) was used in the following experiments.

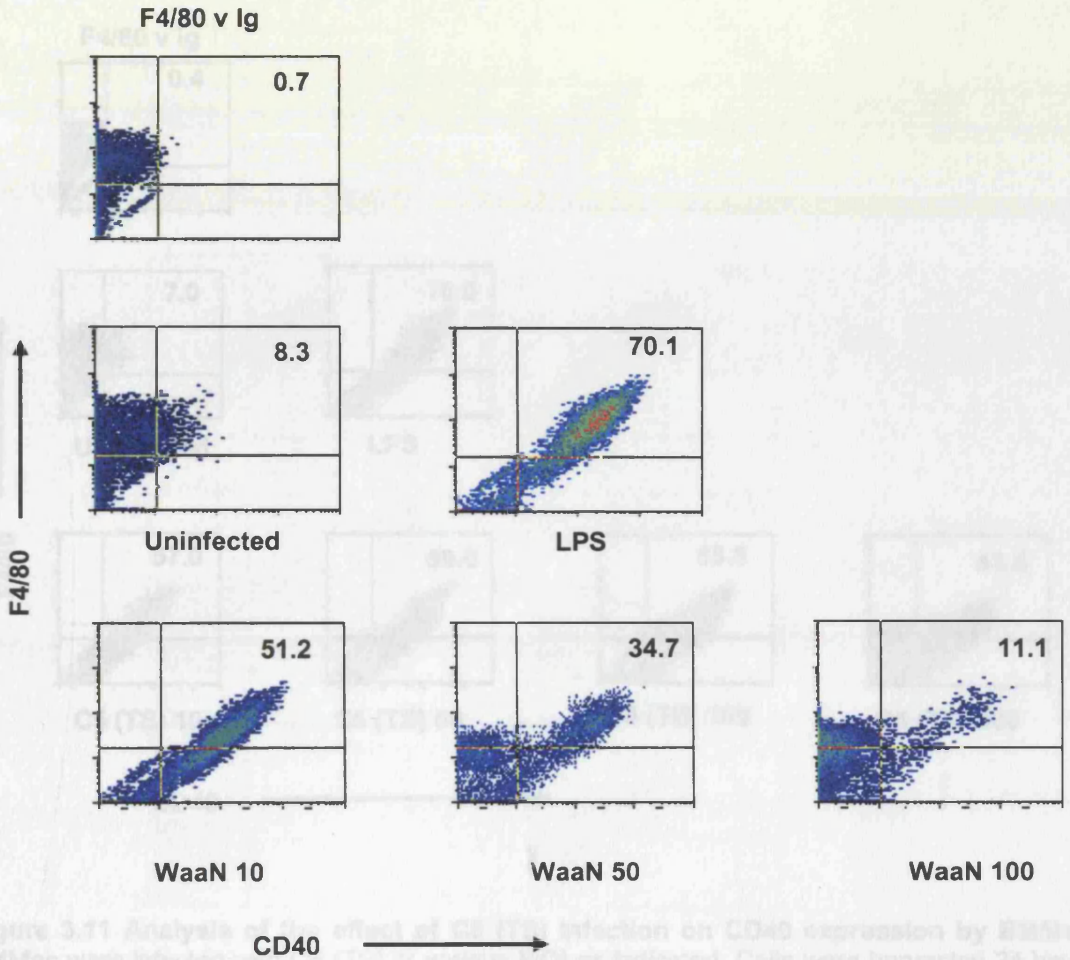


Figure 3.10 Analysis of the effect of waaN infection on CD40 expression by BMMac. BMMac were infected with waaN at various MOI as indicated. Cells were harvested 24 hours post infection and double stained with antibodies against F4/80 (PE) and CD40 (Cy-chrome). The percentage of double positive staining cells is shown on the top right corner of density plot. The plot on the top row is the control of F4/80 versus Ig, from which the quadrant was set. Uninfected cells and LPS treated cells serve as controls for the infection. Data are the representative of 3 experiments.

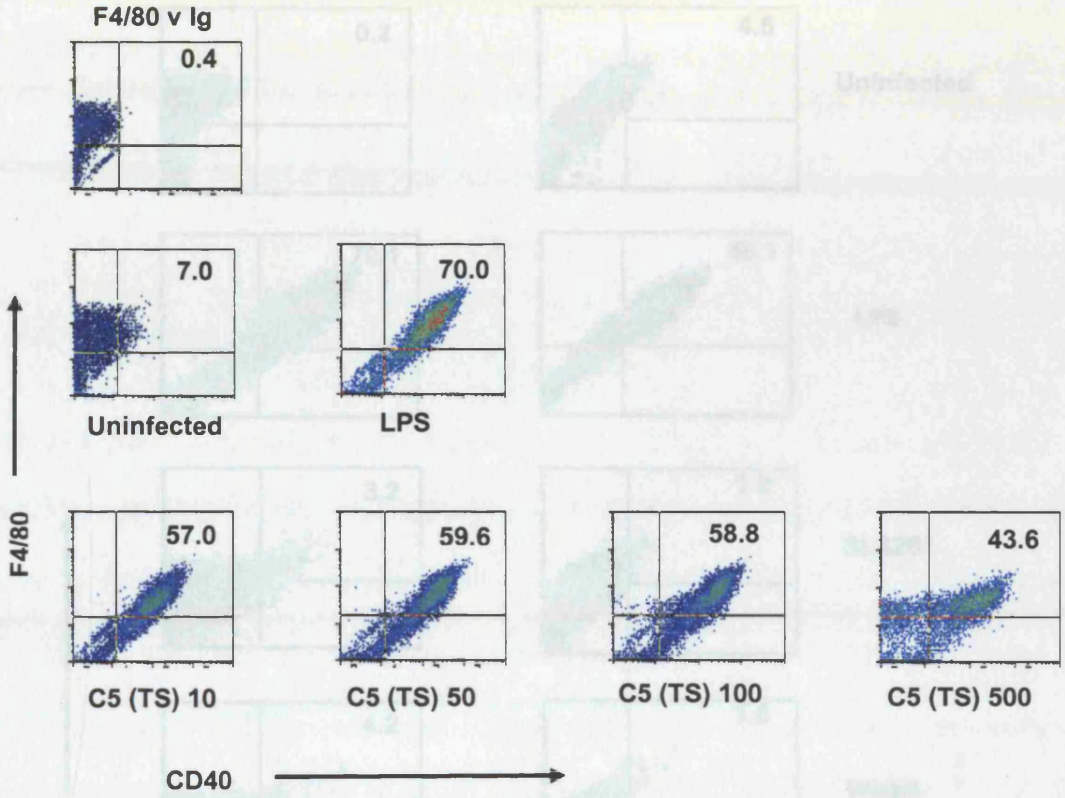


Figure 3.11 Analysis of the effect of C5 (TS) infection on CD40 expression by BMMac. BMMac were infected with C5 (TS) at various MOI as indicated. Cells were harvested 24 hours post infection and double stained with antibodies against F4/80 (PE) and CD40 (Cy-chrome). The percentage of double positive staining cells is shown on the top right corner of density plot. The plot on the top row is the control of F4/80 versus Ig, from which the quadrant was set. Uninfected cells and LPS treated cells serve as controls for the infection. Data are the representative of 3 experiments.

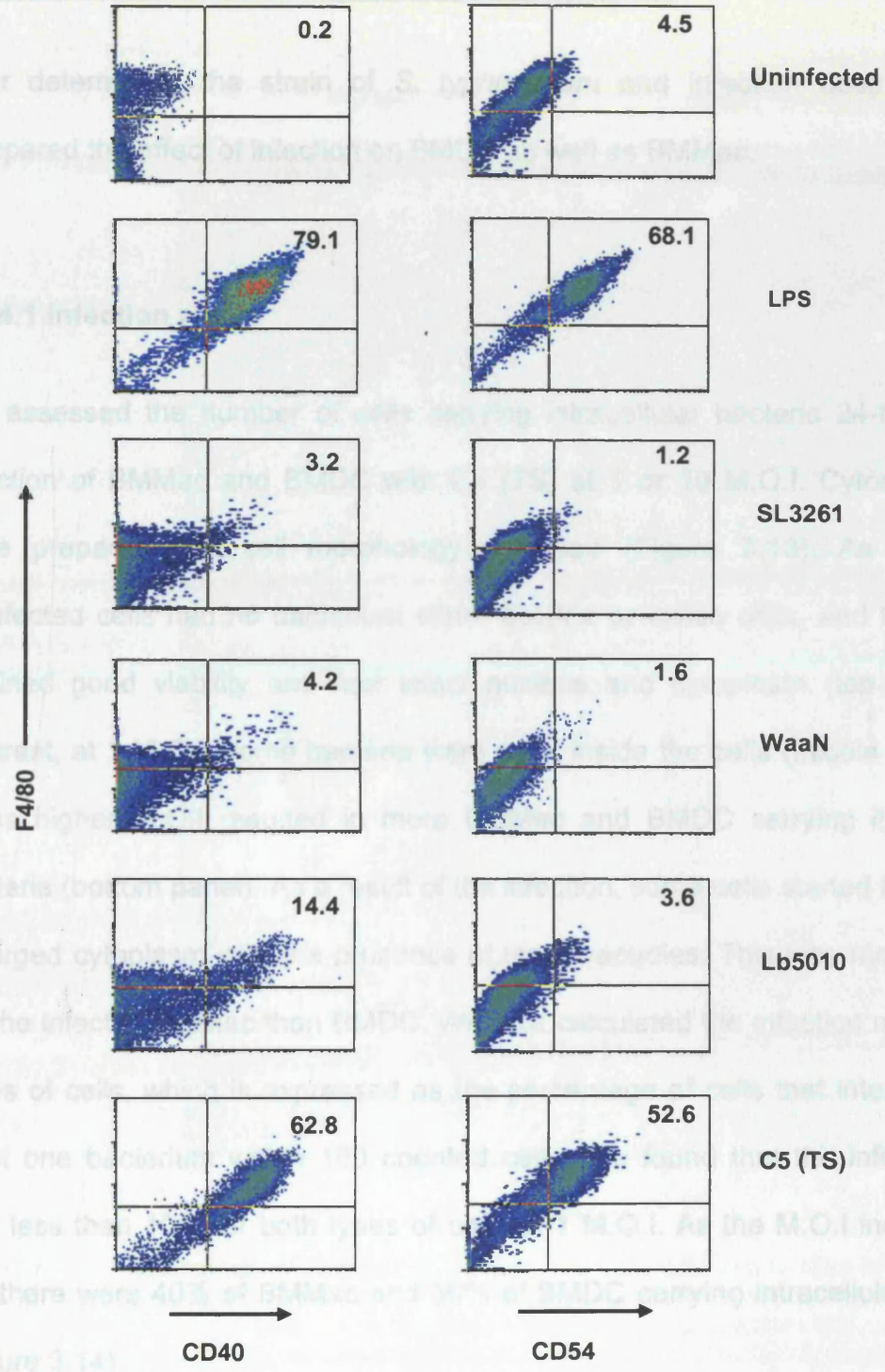


Figure 3.12 Comparison of the effects of infecting BMMac with the 4 strains of *S. typhimurium*. BMMac were infected with 4 strains of *S. typhimurium* as indicated at 10 MOI. Cells were harvested 24 hours post infection and double stained with F4/80 (PE) and CD40 (Cy-chrome) or F4/80 (PE) and CD54 (FITC). Uninfected and LPS treated BMMac serve as controls.

3.2.4 Comparison of the infected BMMac and BMDC

After determining the strain of *S. typhimurium* and infection dose, we then compared the effect of infection on BMDC as well as BMMac.

3.2.4.1 Infection rate

We assessed the number of cells carrying intracellular bacteria 24-hours post infection of BMMac and BMDC with C5 (TS) at 1 or 10 M.O.I. Cytospin slides were prepared and cell morphology analysed (Figure 3.13). As expected, uninfected cells had no bacterium either outside or inside cells, and these cells retained good viability and had intact nucleus and cytoplasm (top panel). In contrast, at 1 M.O.I, some bacteria were seen inside the cells (middle panel). 10 times higher M.O.I resulted in more BMMac and BMDC carrying intracellular bacteria (bottom panel). As a result of the infection, some cells started to show an enlarged cytoplasm with the presence of more vacuoles. This was more evident for the infected BMMac than BMDC. We next calculated the infection rate of both types of cells, which is expressed as the percentage of cells that internalised at least one bacterium within 100 counted cells. We found that the infection rate was less than 10% for both types of cells at 1 M.O.I. As the M.O.I increased to 10, there were 40% of BMMac and 30% of BMDC carrying intracellular bacteria (Figure 3.14).

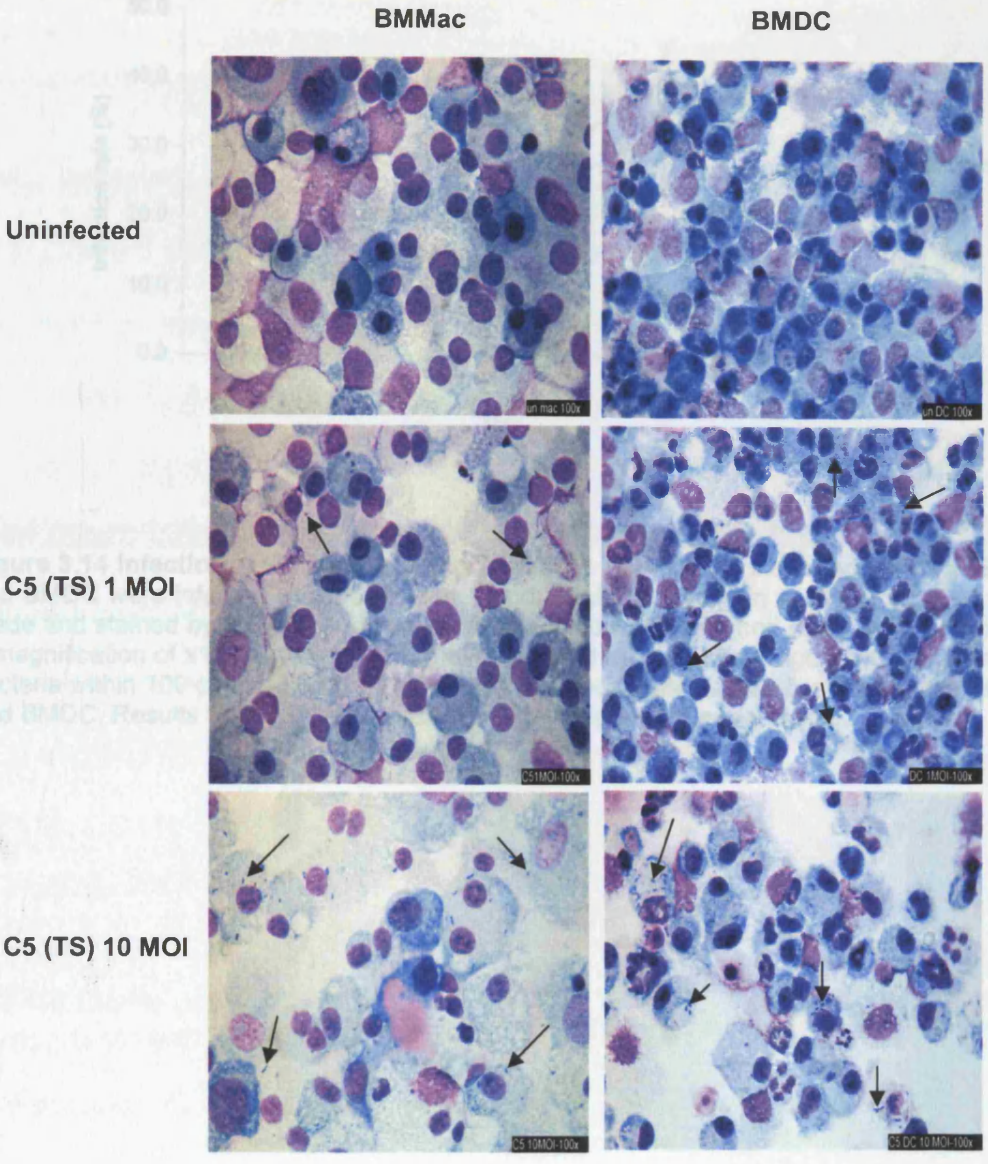


Figure 3.13 Morphological changes of BMMac and BMDC 24 hours after exposure to C5 (TS). BMMac (left panel) and BMDC (right panel) were infected with C5 (TS) at 1 and 10 MOI. Infected cells were harvested 24 hours post-infection and cytospins prepared. Slides were stained with the Diff-Quick staining set. Photographic images were taken at a magnification of x100. Arrows indicate the internalised bacteria.

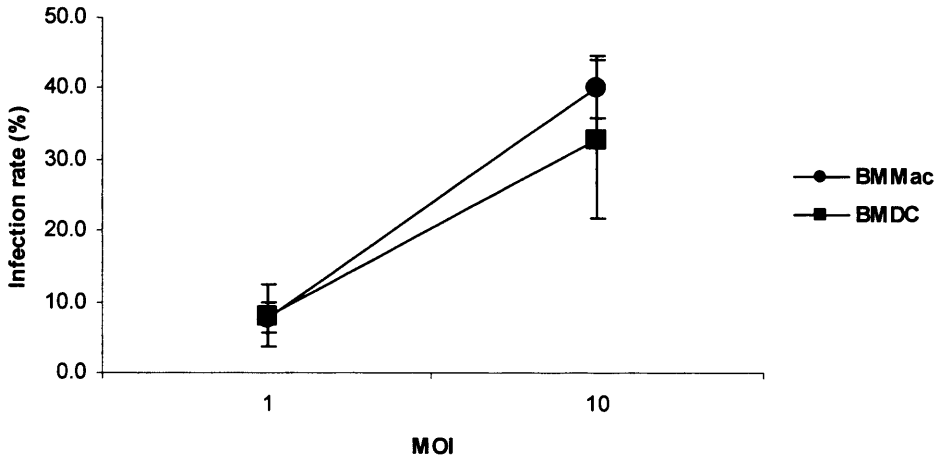


Figure 3.14 Infection rate of BMMac and BMDC 24 hours after exposure to C5 (TS). BMMac and BMDC were infected with C5 (TS) at 1 and 10 M.O.I. Cytospin slides of infected cells were made and stained by Diff-Quick staining set. The slide was examined under a light microscopy at a magnification of x100. The infection rate, expressed as the percentage of cells that internalized bacteria within 100 counted cells in three randomly chosen fields was compared between BMMac and BMDC. Results represent mean value of two independent experiments.

3.2.4.2 Alteration of cell surface molecule expression

The expression of a number of cell surface molecules involved in antigen presentation and co-stimulation of T cells was evaluated by flow cytometry on BMMac and BMDC 24 hours after C5 (TS) infection. Compared to uninfected cells, indicated as medium treated cells (Figure 3.15), LPS treated cells or C5 (TS) infected BMMac showed greatly increased expression of CD40 and CD54, a moderate increase of CD80 and CD86, but no change in expression of either MHC class II I-A/I-E or class I H-2Dd and HLA-B27. However, in contrast to their uninfected counterparts (Figure 3.16), LPS treated or C5 (TS) infected BMDC dramatically increased the expression of CD86, CD80, CD40. Unlike BMMac, the expression of MHC class II I-A/I-E in BMDC was also significantly upregulated. CD54 expression was increased to a moderate extent, and the expression of H-2Dd and HLA-B27 was slightly increased. Infection did not change expression of CD11b, CD11c or F4/80 on either type of cells.

3.2.4.3 Nitrite production

The production of reactive NO was assessed by quantitating the accumulation of NO_2^- in the supernatant of infected cells at 24 hours post infection using the Griess assay. Infection with C5 (TS) at 10 M.O.I induced significant amounts of reactive NO from both BMDC and BMMac, which followed the same trend as that of LPS treated cells (Figure 3.17). More NO was detected from C5 (TS) infected BMDC than BMMac.

3.2.4.4 Secretion of pro-inflammatory cytokines

The production of a range of pro-inflammatory cytokines by BMMac and BMDC in response to C5 (TS) infection was examined by flow cytometry. No cytokines were detected in uninfected either BMMac or BMDC, whereas TNF-α, IL-6, IL-12 (p40) were expressed by LPS treated BMMac and BMDC. IFN-γ was not detected in untreated or treated cells. Upon C5 (TS) infection (10 M.O.I), we found that the majority of BMMac expressed large amounts of TNF-α, IL-6 and IL-12p40. In contrast, only a small proportion of BMMac expressed TNF-α, IL-6 or IL-12p40 after SL3261 infection (Figure 3.18). In BMDC, however, it was observed that both strains induced expression of TNF-α, IL-6 and IL-12p40 upon infection at 10 M.O.I, and that SL3261 did so to a greater extent than to C5 (TS) (figure 3.19). Finally, although a similar pattern of cytokine production was detected on both cell types after 10 M.O.I C5 (TS) infection, overall, BMDC expressed lower amounts of cytokines compared to BMMac.

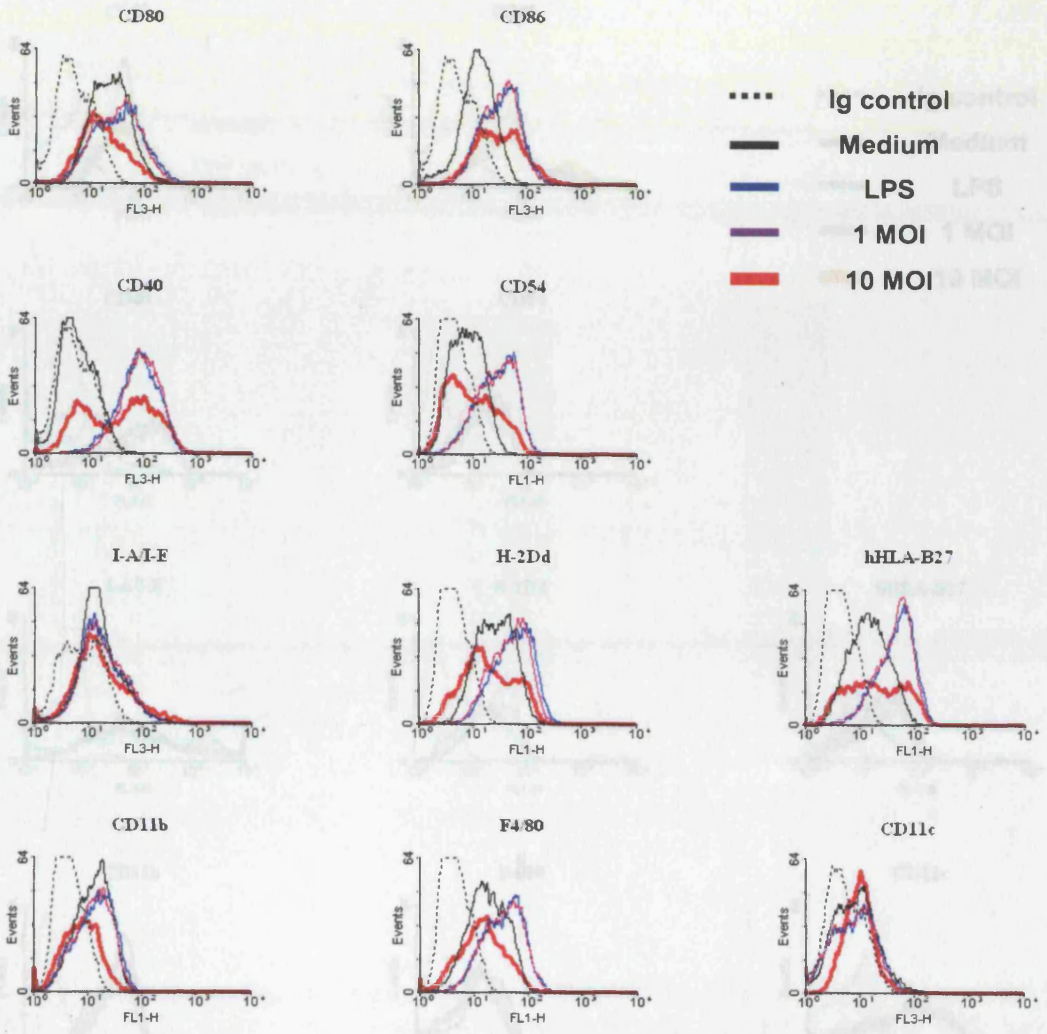


Figure 3.15 Alteration of cell surface marker expression on BMMac after 24 hours exposure to C5 (TS). BMMac were infected with C5 (TS) at 1 and 10 MOI for 2 hours. In parallel, cells cultured in medium or in the presence of 1μg/ml LPS were used as controls. Following infection, remaining bacteria were washed away and the cells cultured for an additional 24 hours in the presence of 50 μg/ml gentamicin. Cells were harvested and stained with antibodies recognizing CD54, H-2D^d, HLA-B27, CD11b, F4/80, CD40, I-A/I-E, CD80, CD86 and CD11c. Histograms show expression of markers of BMMac under different conditions as indicated. Results are representative of two independent experiments.

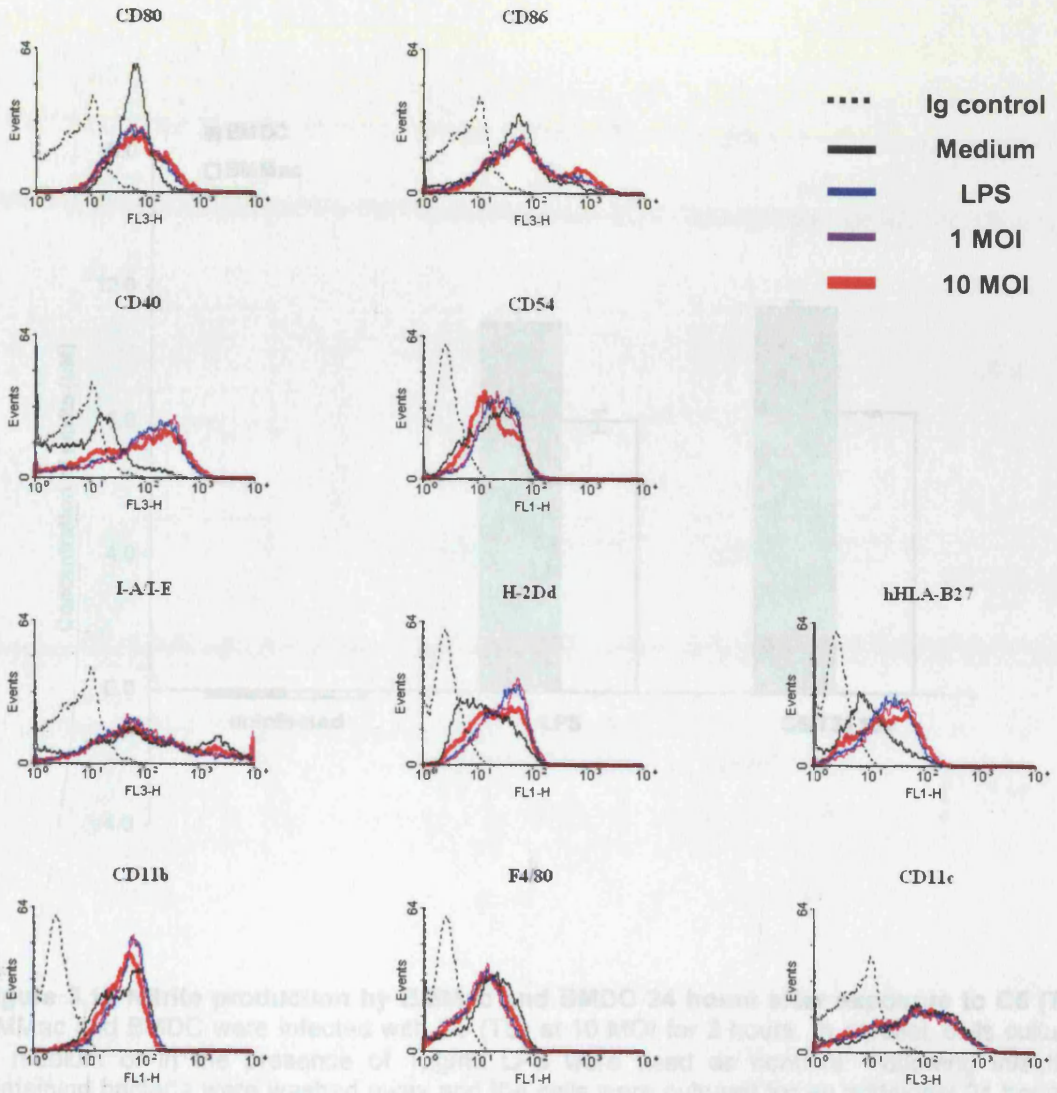


Figure 3.16 Alteration of cell surface marker expression on BMDC after 24 hours exposure to C5 (TS). BMDC were infected with C5 (TS) at 1 and 10 MOI for 2 hours. In parallel, cells cultured in medium or in the presence of 1μg/ml LPS were used as controls. Following infection, remaining bacteria were washed away and the cells cultured for an additional 24 hours in the presence of 50 μg/ml gentamicin. Cells were harvested and stained with antibodies recognizing CD54, H-2D^d, HLA-B27, CD11b, F4/80, CD40, I-A/I-E, CD80, CD86 and CD11c. Histograms show expression of markers of BMDC under different conditions as indicated. Results are representative of two independent experiments.

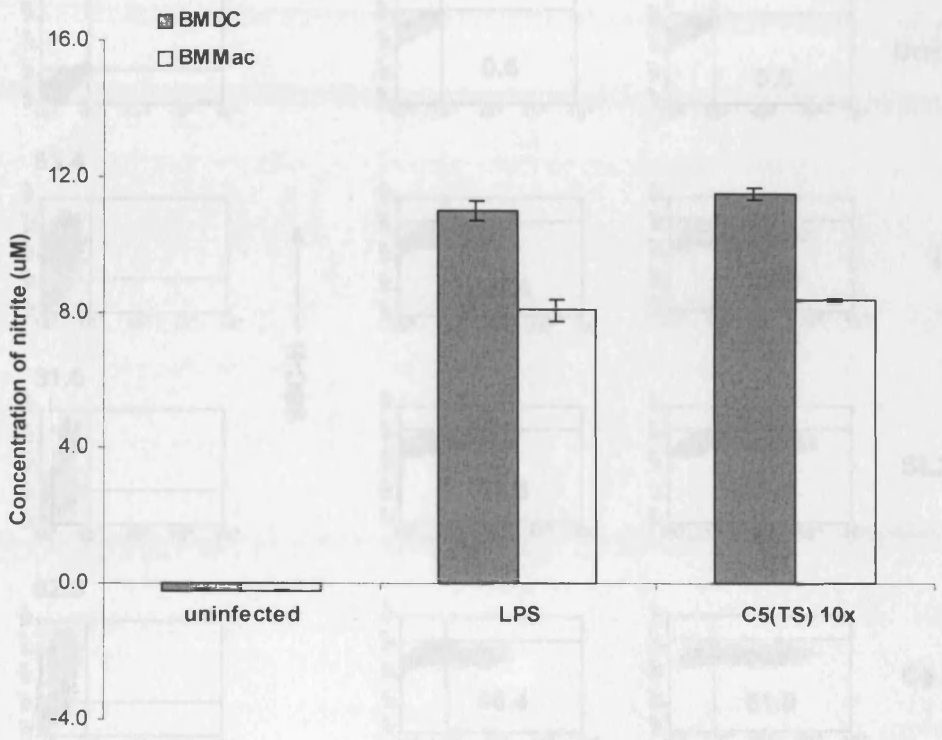


Figure 3.17 Nitrite production by BMMac and BMDC 24 hours after exposure to C5 (TS). BMMac and BMDC were infected with C5 (TS) at 10 MOI for 2 hours. In parallel, cells cultured in medium or in the presence of 1μg/ml LPS were used as controls. Following infection, remaining bacteria were washed away and the cells were cultured for an additional 24 hours in the presence of 50 μg/ml gentamicin. Supernatants of cultures were harvested, and nitrite, which is the stable product of nitric oxide, was measured by the Griess assay. The concentration of nitrite was calculated using a standard curve of sodium nitrite. Results are the mean values ± SD of triplicate for each sample.

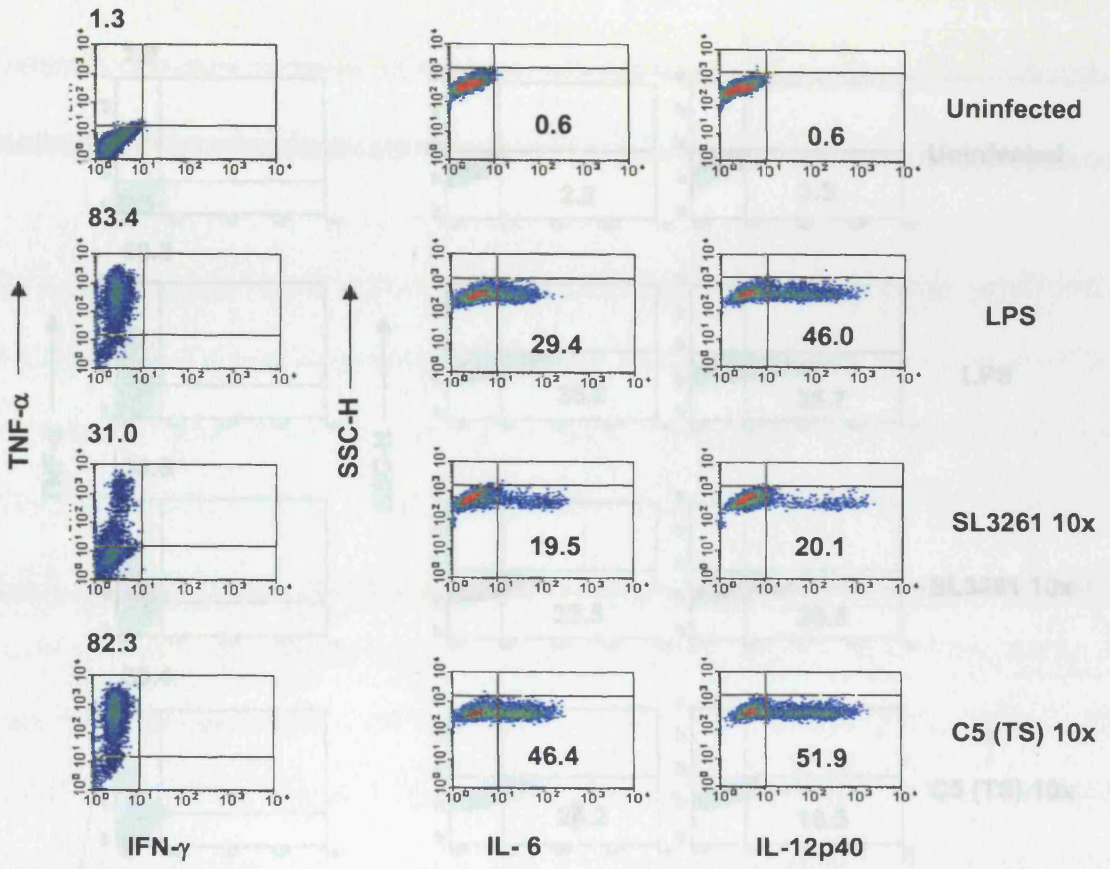


Figure 3.18 Cytokine secretion of BMMac 24 hours after exposure to C5 (TS) or SL3261. BMMac were infected with C5 (TS) or SL3261 at 10 MOI for 2 hours. In parallel, cells cultured in medium or in the presence of 1μg/ml LPS were used as controls. Following infection, remaining bacteria were washed away and the cells were cultured for an additional 24 hours in the presence of 50 μg/ml gentamicin. Cells were harvested and stained with antibodies recognising TNF-α (APC), IL-6 (PE), IL-12p40 (PE) and IFN-γ (FITC). Percentage of cytokine secreting cells is indicated. Data are representative of 3 experiments.

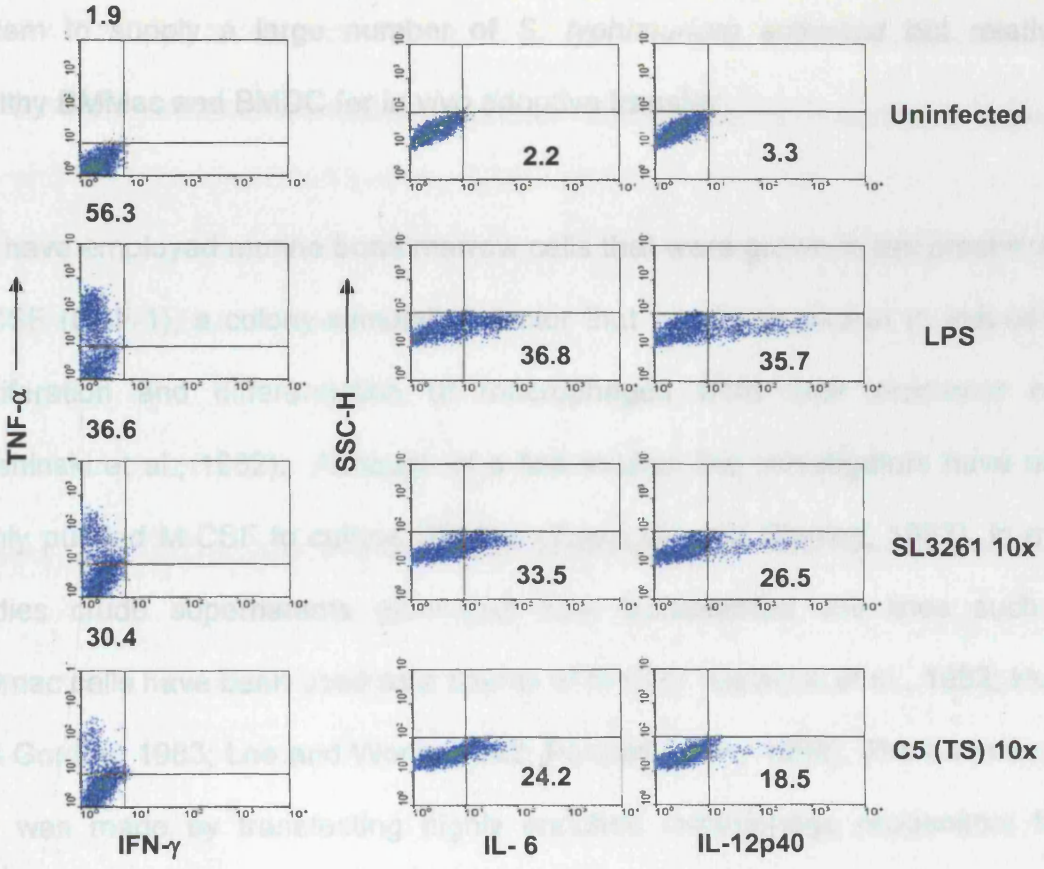


Figure 3.19 Cytokine secretion of BMDC 24 hours after exposure to C5 (TS) or SL3261. BMDC were infected with C5(TS) and SL3261 at 10 MOI respectively for 2 hours. In parallel, cells cultured in medium or in the presence of 1 μg/ml LPS were used as controls. Following infection, remaining bacteria were washed away and the cells were cultured for an additional 24 hours in the presence of 50 μg/ml gentamicin. Cells were harvested and stained with antibodies recognising TNF-α (APC), IL-6 (PE), IL-12 (PE) and IFN-γ (FITC). Percentage of cytokine secreting cells is indicated. Data are representative of 3 experiments.

3.3 Discussion

The aim of this part of study was to set up a reproducible *in vitro* experimental system to supply a large number of *S. typhimurium* activated but relatively healthy BMMac and BMDC for *in vivo* adoptive transfer.

We have employed murine bone marrow cells that were grown in the presence of M-CSF (CSF-1), a colony-stimulating factor that has been shown to induce the proliferation and differentiation of macrophages from their precursor cells (Tushinski et al., 1982). Although in a few studies the investigators have used highly purified M-CSF to culture BMMac (Tushinski and Stanley, 1983), in most studies crude supernatants generated from transformed cell lines such as Ladmac cells have been used as a source of M-CSF (Calamai et al., 1982; Hume and Gordon, 1983; Lee and Wong, 1982; Ponniah et al., 1996). The Ladmac cell line was made by transfecting highly enriched macrophage progenitors from mouse bone marrow cells with a human oncogene, R-myc, therefore resulting in its constitutive secretion of M-CSF that was capable of supporting the *in vitro* proliferation of bone marrow macrophages (Sklar et al., 1985). A previous study used 20% of this supernatant in cultures to supply the required amount of M-CSF (Ponniah et al., 1996). In our study, we quantified the M-CSF contained in the supernatant collected from different batches of Ladmac cell cultures and found that 20% Ladmac cell supernatant was equivalent to 6~9 ng/ml recombinant M-CSF, which was similar to the required concentration of M-CSF (1000~2000U/ml corresponds to 6~12 ng/ml) for yielding a maximum proliferative response in BMMac culture (Tushinski et al., 1982). Furthermore, unlike the short period of

culture for BMMac used in other studies (Calamai et al., 1982) (Pannetier et al., 2004) we cultured BMMac for 3 weeks in order to improve cell yield.

Some reports have shown that the generation of exclusively immature DC by the use of GM-CSF alone (Inaba et al., 1992; Labeur et al., 1999; Lu et al., 1997; Lu et al., 1995), whereas others yielded mature DC by combination of GM-CSF and IL-4 (Garrigan et al., 1996) (Antonysamy et al., 1999; Ildstad and Sachs, 1984; Mayordomo et al., 1997). IL-4 is typically used in combination with GM-CSF for the generation of DC from human monocyte-derived DC, where it inhibits the outgrowth of macrophages (Menges et al., 2005). However, for the generation of murine BMDC, IL-4 is not required to influence the simultaneous outgrowth of macrophages especially when high dose of GM-CSF are used in the culture. Instead, it acts on immature DC to promote their maturation (Lutz et al., 2000) and also enhances their LPS-induced IL-12 production (Hochrein et al., 2000; Lutz et al., 2002). A panel of stimulators such as TNF-α, LPS or CD40 L can also be used to induce further maturation of DC in the later stages of culture (Banchereau and Steinman, 1998; Cella et al., 1997). Thus, in order to yield more immature DC, we chose to use GM-CSF alone for our BMDC culture with the absence of IL-4 or other maturation stimulators. A commercial source of GM-CSF was used due to a lack of a GM-CSF secreting cell line in our laboratory. We adapted our protocol from previously described methods (Inaba et al., 1992; Lutz et al., 1999). It has been documented that GM-CSF can stimulate three distinct types of myeloid cells, i.e. granulocytes, macrophages and DC (Inaba et al., 1992; Lutz et al., 2000). In these studies, three stable populations were generated with GM-CSF: DC and granulocytes in nonadherent or semi adherent

state with macrophages in a firmly adherent state. However, after some major modifications made to the basic protocol applied by those investigators, Lutz and colleagues found that application of a very low seeding density of bone marrow cells (2×10^5/ml) and the reduction of GM-CSF dose (10 ng/ml) at a later stage of culture could dramatically reduce granulocyte contamination (Lutz et al., 1999). We followed their protocol with some further modifications. We found that after 7-days stimulation with GM-CSF, adherent cells expressed comparable levels of the DC marker CD11c on their surface as nonadherent cells (Figure 3.4). These cells have been assumed to be macrophages in other studies without detailed characteristation of this population (Inaba et al., 1992; Lutz et al., 1999). Furthermore, by harvesting solely adherent populations, we further reduced the contamination of granulocytes to less than 10% of cells expressing Gr-1 (20% of nonadherent cells expressing Gr-1). More importantly, compared to non-adherent cells, adherent cells showed a more immature phenotype illustrated by the greater proportion of cells expressing low levels of MHC class II (Figure 3.4), which was also observed in other studies (Menges et al., 2005; Peche et al., 2005; Vecchione et al., 2002) regardless of species. Given all these reasons, instead of the nonadherent population, we harvested the adherent population of our DC culture. Adherent BMDC have been chosen to be used in studies such as that by Muthana and colleagues (Muthana et al., 2004) although the nonadherent population of BMDC is generally used (Fischer et al., 2001; Lutz et al., 1999). One of the aims of our BMDC culture was to generate immature DC before infection with *S. typhimurium* and to have large numbers for adoptive transfer into mice. Unfortunately, discarding nonadherent populations from our DC culture resulted in wasting more than half the population of CD11c (+) DC. In order to

enrich nonadherent cells and achieve a maximal purity of BMDC culture, we employed tissue culture flasks instead of petri dishes to grow the cells, which allowed more space for cells to grow in an adherent state. Muthana and colleagues also employed tissue culture flasks for propagating their adherent BMDC (Muthana et al., 2004). After 7-days culture using this approach, we found that two thirds of cells generated were adherent.

The day of harvesting the DC culture can influence the maturation state of DC (Lutz et al., 1999). We found that harvesting DC at day 5 also led to a lower yield (Table 3.2). Although Lutz et al found that BMDC purity and yield could be further enhanced if the culture was extended until day 10~12, many of these BMDC might have reached their final stage of maturation and thus started to show signs of apoptosis (Lutz et al., 1999). More importantly, they found that the optimal stimulation of allogeneic T cells was on days 8~10. Thus to obtain a balance of immature DC with a good yield of cells, day 8 was chosen as the harvest day to maintain a high cell yield and optimal T cell stimulation capacity.

Phenotypically, we distinguish BMDC from BMMac by their different myeloid cell surface marker expression. BMDC were defined as CD11b (+) CD11c (+), whereas BMMac were CD11b (+) CD11c (-) (Figure 3.6 and 3.7). This definition was also applied by Macpherson and colleagues to sort their DC and macrophages from mesenteric lymph nodes or Peyer's patches (Macpherson and Uhr, 2004). The overall characterisation of these two types of cells is summarised in Table 3.4.

Table 3.4 Comparison of cell surface molecules expression on BMDC and BMMac

Molecule	**BMDC**	**BMMac**
CD11b	(+++)	(++)
CD11c	(+++)	(-)
F4/80	(+)	(++)
Class II	(++)	(-)
Class I	(+++)	(+++)
HLA-B27	(+)	(+)
Gr-1	(-)	(-)
CD3	(-)	(-)
B220	(-)	(-)

However both BMMac and BMDC expressed F4/80, an established marker for macrophages (Sadahira and Mori, 1999). F4/80 was also found consistently at low levels on BMDC generated by principle DC protocols (Inaba et al., 1992; Lutz et al., 1999), which revealed that F4/80 is not specifically expressed by macrophages. So far macrophages have no distinctive cell-surface antigen that allows for their enrichment (van Furth, 1992). Thus, although we used F4/80 as a marker for macrophages, it is important to be aware that F4/80 cannot be used to distinguish BMMac from BMDC.

Mouse MHC class II I-A/I-E was not expressed by BMMac, but was expressed by BMDC, further supporting the claim that these were two distinct populations. Furthermore, the class II expression on BMDC was heterogeneous, as shown in Figure 3.7, two subpopulations could be detected by their distinct level of MHC class II expression, the MHC class II^{low} cells referred to as immature DC compared to MHC class II^{high} cells referred to as mature DC. Such heterogeneous expression on BMDC cultures is an agreement with other studies (Cheminay et al., 2002; Inaba et al., 1992; Lutz et al., 1999).

Unlike class II molecules, MHC class I molecules are expressed on nearly all tissue cells, and in this study, we found that BMDC and BMMac both expressed the mouse MHC class I molecule-H-2Kd. HLA-B27 was also expressed by both types of cells as the bone marrow was derived from HLA-B27 transgenic mice. Modifications to the cultures also ensured a low number of contaminating Gr-1 (+) granulocytes in the cultures. The absence of B220 (+) and CD3 (+) cells also confirmed the purity of the culture; the use of adherent populations in both

cultures ensured a lower level of contaminating cells since granulocytes, T and B cells all grow in a nonadherent state.

In summary, highly enriched cultures of macrophages and DC were generated from the bone marrow of HLA-B27 transgenic mice and could be grown in large numbers for all subsequent experiments.

S. typhimurium is a causative organism for ReA accounting for 60% of *Salmonella*-induced ReA cases (Maki-Ikola O, 1991). Consequently, it is imperative to know what effect *S. typhimurium* has on the immune system and, specifically, its effect on the cells it invades, i.e. macrophages and/or DC. To investigate this, BMMac and and BMDC were infected, *in vitro*, with several attenuated strains of *S. typhimurium* at different M.O.I, and the effect on expression of cell surface molecules analysed. The reason for use of the attenuated strain is that infection with such strains in mice causes an analogous pathology to that observed in man caused by the wild type *S. typhimurium*. Since *S. typhimurium* induced gastroenteritis can trigger ReA in humans, inoculation of attenuated *S. typhimurium* in mice serves a good model for the study of ReA in humans.

It was important to establish an infection protocol that led to a good rate of infected cells that remained in an activated but healthy state. Firstly, we had to choose the state of bacterial growth. Bacteria in logarithmic growth are more active, viable and have increased invasive ability as compared to stationary phase bacteria. In previous work in our lab, when stationary phase of bacteria

were used to infect macrophages, the maximal infection rate was only 10%. We found that the infection rate using mid-log phase bacteria (180 minutes) enhanced infection rate up to approximately 50%.

We then set out to select the optimal strain and infection conditions using BMMac. Experiments for optimisation of strains and titration of M.O.I revealed that SL3261 and waaN were not suitable agents for our experiments, since even at low M.O.I (10), cells showed poor signs of activation. This was due to the fact that cells were killed to a much greater extent by these trains than by Lb5010 and C5 (TS) (Figure 3.9-3.12). SL3261 and waaN infected cell death were also reflected by the profiles of FSC vs SSC and further confirmed by cytospin preparations (data not shown).

The apparent cytotoxicity of SL3261 and waaN strains to macrophages at high M.O.I is not unexpected. Using time-lapse video microscopy, it has been found that cytotoxicity caused by *Salmonella* is initially manifested by inhibition of membrane ruffling and macropinocytosis in infected macrophages, followed by cell death (Chen et al., 1996a). It was also reported that *Salmonella* cytotoxic effects are strictly dependent upon the expression of invasion-associated Type III protein-secretion (Hersh et al., 1999; Monack et al., 1996). The 4 strains of *Salmonella* we used are all attenuated in different ways. SL3261 and waaN are mutants in aroA (auxotrophic) or waaN (catalyse lipid A biosynthesis) genes respectively, which encode proteins that are not required for bacterial entry into cells, so they remain cytotoxic to macrophages. In contrast, Lb5010 is a poorly invasive mutant, which may somewhat impair its cytotoxic ability and result in

less macrophages being killed. For C5 (TS), it is possible that the 2-hour infection of macrophages and bacteria at 30°C restricts the optimal expression of its TTSS. Consequently, infected cells keep a healthy appearance in the presence of infection at high M.O.I. Together with its ability to persist in peripheral areas *in vivo* and readily cause arthritis, we decided to use this strain of *S. typhimurium* to carry on the following experiments

In terms of the infection rate, our data suggested that under the same infection conditions, there are more BMMac (40%) compared to BMDC (30%) infected with *S. typhimurium* resulting in more *S. typhimurium* residing inside the infected cells (Figure 3.13). However, it was reported that when bovine monocyte-derived macrophages and DC were infected with *S. typhimurium* at a M.O.I of 10, there was only approximately 10% of infected cells carrying intracellular bacterium, and no difference was detected between macrophages and DC regarding of the number of bacteria recovered intracellularly (Norimatsu et al., 2003). This discrepancy may due to the application of different protocols in determination of the infection rate. In their study, they lysed infected cells and then calculated the CFU, according to the number of cells put into infection and figured out the infection rate. In contrast, we counted the visible bacteria inside the cells to obtain the infection rate on cytospin preparation from infected cells. Notably, we also found that there were some cells with numerous intracellular bacteria and some cells with just one engulfed bacterium (Figure 3.13), which again would not be reflected in their protocol. Furthermore, it was suggested that these intracellular *S. typhimurium* may not necessarily represent a static population, and their fate may differ depending on whether they were up taken by BMDC or

BMMac. Indeed, it has been demonstrated that after infection of RAW264.7, a macrophage cell line, a rapid proliferation of wild-type *S. typhimurium* was observed. This proliferation required virulence genes encoded by SPI-2, since a mutant strain deficient in SPI-2 function did not replicate. In contrast, the data of the fate of intracellular *S. typhimurium* inside DC remain controversial, although SPI-2 mediated virulence factors were also activated in BMDC, no increase of intracellular bacteria, either wild type or SPI-2 deficient, was observed in infected BMDC, indicating that intracellular *S. typhimurium* in BMDC were a non-dividing population (Jantsch et al., 2003). However, another study also claimed that not only could *S. typhimurium* survive within DC, but some replication of these bacteria could also occur intracellularly, as has been observed for cultured macrophages (Marriott et al., 1999a). Further studies about the fate of intracellular *S. typhimurium* inside DC and macrophages will be required.

We have shown that BMMac and BMDC were both activated by C5 (TS) infection, reflected by upregulation of a number of cell surface molecules (Figure 3.15 and 3.16), which is consistent with the findings of others (Yrlid et al., 2000). Firstly, in response to C5 (TS) infection, we found that BMMac greatly increased CD40 and CD54, whereas, BMDC markedly increased CD80, CD86 and MHC-II but to lesser extent increased CD40 and CD54. Low-dose *S. typhimurium* infection has also been shown to induce a marginal increase in CD40 on BMMac with no detectable increase in expression of the other surface molecules (Norimatsu et al., 2003). CD40 and CD40 ligand belong to the TNF family of receptor and ligands. These molecules play a role in stimulating the T-cell response, and CD40 is also involved in macrophage and B cell activation.

Previous studies have demonstrated that *in vitro* treatment of *Salmonella*-infected macrophages from BALB/c mice with soluble trimeric CD40 ligand results in elevated production of interleukin 12p70 by these cells, suggesting CD40-CD40 ligand interactions are significant events in the protective response against this intracellular pathogen in mice (Marriott et al., 1999b). A heightened susceptibility to intracellular pathogens is observed in CD40L-/- mice, which is associated with impaired macrophage activation and an altered inflammatory response, as reflected by low levels of TNF, NO (Soong et al., 1996) and IL-12 production (Campbell et al., 1996). These studies in combination with our results showing that BMMac expressed higher levels of inflammatory cytokines compared to BMDC upon *S. typhimurium* infection suggest that macrophages contribute to the first line against *S. typhimurium* invasion to a greater extent than DC.

CD54, also known as intercellular adhesion molecule 1 (ICAM-1), binds to T cell integrin LFA-1, which stabilizes the association between the antigen-specific T cell and antigen-presenting cell. Together with other members of same family, such as ICAM-2 and ICAM-3, it also plays part in recruitment of immune cells to inflammatory sites, especially it is used for circulating monocytes to navigate out of vessels and enter peripheral tissue sites. Therefore, significant upregulation of CD54 expression on BMMac in response to C5 (TS) infection is not only an indicator of macrophage activation but also contributes to their emigration from site of infection, which could also be utilized by intracellular bacteria as a vehicle to facilitate their dissemination. Therefore, activation of macrophages upon *S. typhimurium* infection could be a double-edged sword.

On the other hand, costimulatory molecules CD80 (B7-1) and CD86 (B7-2) together with MHC molecules are involved in antigen presentation and stimulation of T cells. Compared to a moderate increase of their expression on BMMac, they were dramatically elevated by BMDC after C5 (TS) infection (Figure 3.16). Selective upregulation of costimulatory molecules by BMDC is consistent with their more potent ability to stimulate T cell responses. This issue will be further discussed in chapter 6. BMMac failed to increase expression of either MHC class I or class II. Rather, some downregulation of class I molecule H-2D^{d} was observed. In striking contrast, class I (H-2Dd and HLA-B27) and class II I-A/I-E were both upregulated on BMDC. These differences point to further differences in antigen presentation capacity. These results are consistent with other reports that co-incubation of BMDC or BMMac with *S. typhimurium* results in upregulation of a number of cell surface molecules (Niedergang et al., 2000; Norimatsu et al., 2003; Yrlid et al., 2000). Furthermore, it has been demonstrated that such upregulation of cell surface molecules does not require internalisation of the bacteria, nor does it require that the bacteria are viable, since similar upregulation of the surface molecules is observed upon co-culture of BMDC or BMMac with *S. typhimurium* in the presence of CCD, an inhibitor of phagocytosis or with heat-killed bacteria (Svensson et al., 2000; Yrlid et al., 2000). In the latter study, it was shown that PhoP, the gene involved in cell invasion encoded by the SPI-1, does not appear to influence surface expression of molecules by BMDC (Svensson et al., 2000).

Co-incubation of BMMac or BMDC with LPS resulted in effects on surface molecule expression similar to those observed with intact bacterium, that is, a

shift from a population with heterogeneous levels of expression of to a more uniform population expressing a similar, high level of some molecules, such as CD40 on BMMac (Figure 3.15). This also confirmed that bacterial viability is not required for *S. typhimurium*-induced activation of both BMMac and BMDC.

NO is one of the radicals belonging to the RNS, together with ROS, generated by the NADPH oxidase and iNOS (MacMicking et al., 1997). The production of RNS and ROS by phagocytes is of particular significance in resistance to intracellular pathogens (Vazquez-Torres et al., 2000a). We have shown that both BMMac and BMDC produced significant amounts of NO in response to C5 (TS) infection (Figure3.17). NO was also produced by LPS treated cells. Additionally, we found that there was a greater amount of NO generated by BMDC compared to BMMac in response to either C5 (TS) infection or LPS treatment. NO production by macrophages has been extensively demonstrated and accepted as a major innate defence mechanism to restrict bacterial replication as discussed above (Vazquez-Torres et al., 2000a). However, the production of NO from BMDC in our study was not in line with another report that shows BMDC are poor producers of NO after *S. typhimurium* infection whereas macrophages are robust inducers of NO (Yrlid et al., 2000). On the contrary, other studies have shown that murine DC greatly enhance the expression of iNOS and NO production after ingestion of *Salmonella*, indicating that DC may also participate in the innate defence against intracellular bacteria (Eriksson et al., 2003; Muthana et al., 2004; Norimatsu et al., 2003). The reasons underlying these conflicting results are unclear, although the use of different populations of DC (adherent vs adherent),

DC at different maturational stages or application of different protocols might influence the NO production against *S. typhimurium*.

We also showed that C5 (TS) infection of both BMDC and BMMac resulted in increased production of a range of cytokines, including TNF-α, IL-6 and IL-12p40 but not IFN-γ (Figure 3.18 and 3.19), which was demonstrated by other reports (Norimatsu et al., 2003; Yrlid et al., 2000). Of note, we found that selectively secreted large amount of TNF-α but only small quantities of IL-12p40 and IL-6 BMMac upon C5 (TS) infection. In comparison, overall lower levels of these cytokines were detected after C5 (TS) infection of BMDC. The reduced level of cytokine secretion by BMDC may due to the shortened incubation time after addition of brefeldin A during performance of intracellular cytokine staining, since we applied overnight incubation (12 hours) for BMMac but 6 hours for BMDC before we detected any cytokine expression. However, it is also possible that these two types of cells exhibit inherent difference in cytokine production after *S. typhimurium* infection. Although we did not test IL-1β production, it has been reported that IL-1β is also produced by both types of APCs upon *S. typhimurium* infection at a level similar to other cytokines (Marriott et al., 1999a; Norimatsu et al., 2003).

The detection of cytokine production after *S. typhimurium* infection suggested that both cells participate in initiating a proinflammatory response. Previous studies using CCD treatment and green fluorescent protein (GFP) have shown that although CCD treatment leads to partial inhibition of cytokine production, significant cytokine production by both types of cells occurs in the absence of

bacterial internalization, since a major fraction of cytokine producing cells were GFP negative, did not contain internalised bacteria (Yrlid and Wick, 2002). In our study, we also observed a similar effect when we studied the cytokine response of BMDC using GFP *S. typhimurium* (data not shown). Four fractions of cells were present in the coculture of DC and GFP *S. typhimurium*. A: GFP(+) cytokine(+), B: GFP(-) cytokine(-), C: GFP(+) cytokine(-) and D: GFP(-) cytokine(+). B and C represent cell populations that failed to activate cytokine production even when they were invaded. These cells perhaps have succumbed to the virulence factor secreted by bacteria such as SPI-2 encoding genes. More interestingly, the existence of D population suggests that cell contact-independent soluble components of bacteria also contribute to overall cytokine production consistent with the finding that LPS stimulation also dramatically induced significant amounts of cytokine production from both types of cells (Figure 3.18 and 3.19). Interestingly, it has been previously reported that although bacterial viability is not absolutely required for cytokine production by infected BMDC, the amount of cytokine detected is significantly enhanced by co-incubation of APC with viable bacteria (Yrlid et al., 2000). This is in contrast to the case for infected BMMac, which produce approximately the same amount of cytokines using heat-killed or viable bacteria.

Previous studies of IL-12p40 production after *S. typhimurium* infection have yielded contradictory results. Norimatsu and colleagues found that after exposure to *S. typhimurium*, IL-12p40 was released from infected BMDC but not from BMMac, whereas IL-10 expression was upregulated in BMMac but not BMDC (Norimatsu et al., 2003). This discrepancy may due to the low dose of *S.*

typhimurium (0.001 M.O.I) used in their study of bovine cells, whereas we used 10 M.O.I infection in our mouse model. In addition, different protocols were applied in their and our studies, which may also play a part in generation of these different results. IFN-γ was suspected to be produced by activated macrophages in our early studies of T cells responses, assessed by ELISpots assay. In addition, during the primary response of oral *Salmonella* infection, splenic macrophages are the main population producing IFN-γ (Kirby et al., 2002). Based on these observations, we tested IFN-γ production on *S. typhimurium* infected macrophages and DC using intracellular cytokine staining. However, no IFN-γ was detected in either BMMac or BMMac in response to C5 (TS) infection or LPS stimulation *in vitro*.

3.4 Conclusion

We have established an optimal *in vitro* experimental system for supplying attenuated *S. typhimurium* strain C5 (TS) infected BMMac and BMDC in large numbers. Increased expression of a numbers of cell surface molecules together with several inflammatory cytokines and NO were observed. The fact that these activated cells carried many intracellular bacteria but remained healthy in appearance was also achieved. These infected cells are suitable to be used for adoptive transfer into mice to investigate their migration *in vivo* in the following chapter.

Chapter 4: Migration of *S. typhimurium* infected BMDC and BMMac

4.1 Introduction

In the mouse oral infection model, *S. typhimurium* invades the intestinal mucosa mainly through M cells, colonizes the Peyer's Patches, spreads to the mesenteric lymph nodes and finally disseminates to the spleen and liver (Jones and Falkow, 1996). In addition, it has been shown that CD18-expressing phagocytes including DC and macrophages are involved in an M-cell-independent pathway that permits bacteria transportation across the intestinal epithelium (Vazquez-Torres et al., 1999). Although the interaction of *S. typhimurium* with APCs such as DC and macrophages *in vitro* has been extensively demonstrated (Chen et al., 1996a; Monack et al., 1996; Yrlid et al., 2000; Yrlid and Wick, 2000), the *in vivo* migration pattern of those cells in response to *S. typhimurium* infection has not been addressed. Using a system in which bone marrow derived APCs are infected *in vitro* and then their migration is examined *in vivo* after adoptive transfer into mice will allow the migratory patterns of APCs post *S. typhimurium*-infection to be studied.

S. typhimurium infection can trigger ReA (Hannu et al., 2002), and *Salmonella* antigens such as LPS have been detected in synovial cells of patients with ReA (Granfors et al., 1990). In patients with early stage *Salmonella* infections, *Salmonella* could be identified in peripheral blood monocytes (Kirveskari et al., 1998), suggesting that monocytes play a role in transporting *Salmonella* antigens to the joints of patients with ReA, although, convincing data to support

this assumption are still lacking. Additionally, persistent production of anti-*Salmonella* antibodies of IgM, IgG, and IgA classes is known to be present in *Salmonella*-triggered ReA (Maki-Ikola et al., 1991), indicating persistence of *Salmonella* or components in the arthritic patients. It has been suggested that HLA-B27 impairs the elimination of *Salmonella* within the host cells (Laitio et al., 1997; Virtala et al., 1997), which may offer an explanation for the persistence of ReA-triggering microbes in susceptible HLA-B27 individuals. HLA-B27 restricted CD8 T cells with ReA recognising *Salmonella* have also been identified in synovial fluids of patients (Hermann et al., 1993).

In order to provide insight into the pathogenesis of ReA, we have utilised an HLA-B27 transgenic mouse and *Salmonella* infection system to examine whether *Salmonella*-infected BMMac and BMDC can migrate to inflamed joints. After characterisation of DC and macrophages infected with *S. typhimurium in vitro*, their *in vivo* migration was subsequently investigated by adoptive transfer of fluorescently-labelled cells into HLA-B27 transgenic mice. The presence of labelled cells in non-lymphoid and lymphoid tissues was examined 2 days after transfer under different conditions.

4.2 Results

4.2.1 Migration profile of BMDC and BMMac after C5 (TS) infection

4.2.1.1 Migration profile of BMDC

To investigate the *in vivo* migration profile of BMDC in response to C5 (TS) infection, BMDC were infected with C5 (TS) at 10 M.O.I, with uninfected cells serving as control. Cells were harvested at 24 hours post-infection and labelled with the fluorescent dye CFSE prior to adoptive transfer. Approximately 5×10^6 CFSE labelled cells per mouse were injected intraperitoneally. Single cell suspensions were obtained from various non-lymphoid and lymphoid organs 48 hours post-injection and the CFSE labelled cells (FL1+) were detected by flow cytometry, using cells from a mouse that received no injection of CFSE+ cells as a control to set the CFSE+ region gate. An example of the appearance of recovered cells from the spleen of a recipient mouse is displayed in Figure 4.1A. The number of labelled cells appearing in R1 (CFSE+ cells) after 2×10^6 total cells were collected was compared between mice receiving uninfected or C5 (TS) infected BMDC. Compared to uninfected cells, the number of C5 (TS) infected BMDC was significantly reduced in the peritoneal lavage (PL) (Figure 4.1B). On the contrary, the number of C5 (TS) infected BMDC was significantly increased in the spleen compared to the number of uninfected cells (Figure 4.1C). This was also observed in MLN (Figure 4.1D). The number of uninfected and C5 (TS) infected BMDC was similar in non-lymphoid organs such as the liver and lung (Figure 4.1E, F), and other lymphoid tissues such as inguinal LN (ILN), popliteal LN (PLN) and Peyer's Patches

A

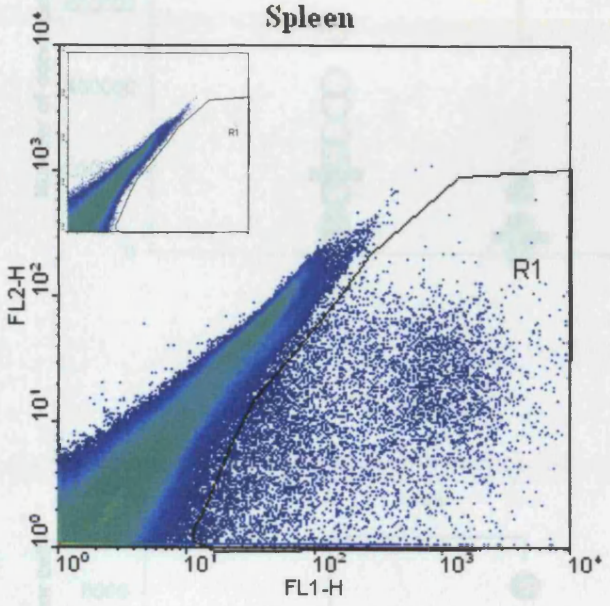
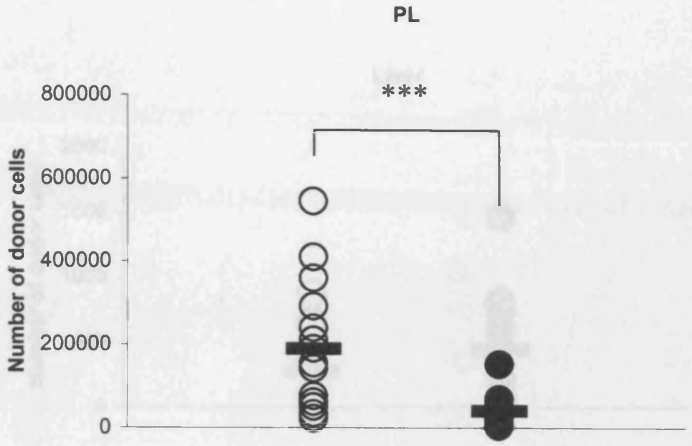
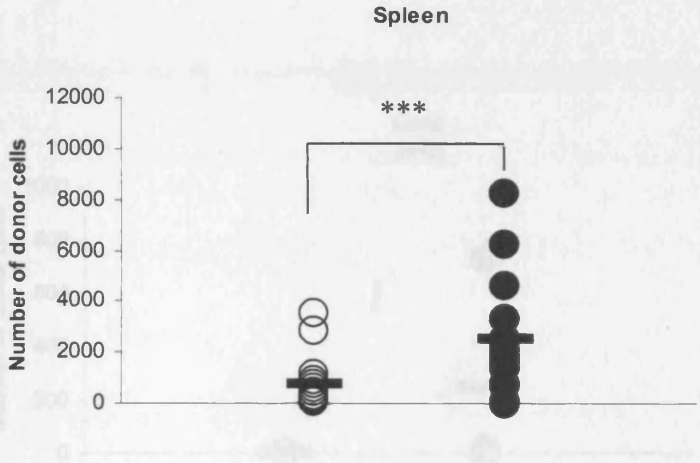


Figure 4.1 (A-I) *In vivo* migration profile of C5 (TS) infected BMDC compared to uninfected BMDC. Uninfected and 10 MOI C5 (TS) infected BMDC were labelled with CFSE. The labelled BMDC were subsequently adoptively transferred into mice by intraperitoneal injection. Forty-eight hours later, spleen, liver and lung were digested with digesting media containing collagenase (1 mg/ml) and DNase (0.5 mg/ml); lymph nodes (LN) and PPs (Peyer's Patches) were mashed with the edges of two frosted slides. Numbers of labelled cells were calculated within 2×10^6 cells collected for each sample by flow cytometry. (A) An example of how the number of labelled cells was derived from the spleen of a recipient mouse. Inset shows a control mouse without transfer, from which the region was set. (B-I) Empty circles (o) indicate uninfected DC and solid circles (•) indicate C5 (TS) infected BMDC. Each bar represents the mean value of 15 animals unless specifically indicated. Data are pooled from 5 independent experiments and statistic significance was analysed using one-way ANOVA. Number of transferred cells recovered from PL (peritoneal lavage)(B), spleen (C) and MLN (mesenteric LN) (D), liver (E) and lung (F), ILN (inguinal LN)(G), PLN (popliteal LN)(H) and PPs (I). ***, $p < 0.01$.

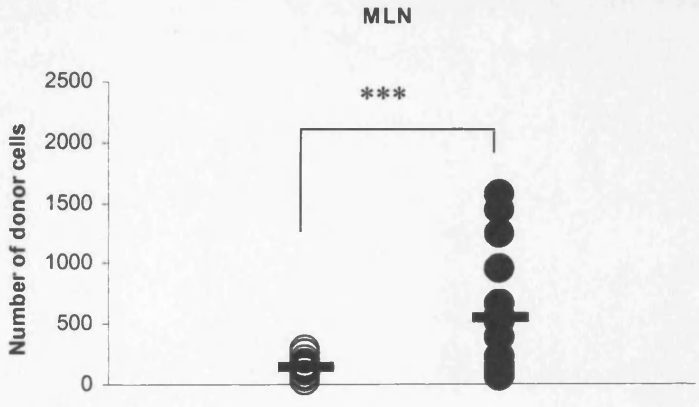
B


PL
***
800000
600000
400000
200000
0
Number of donor cells

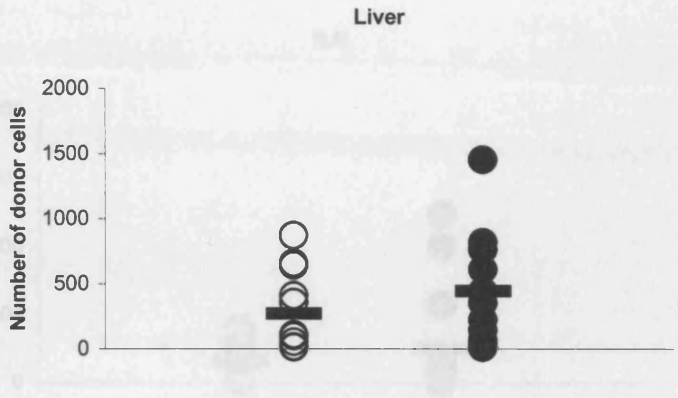
C


Spleen
***
12000
10000
8000
6000
4000
2000
0
Number of donor cells

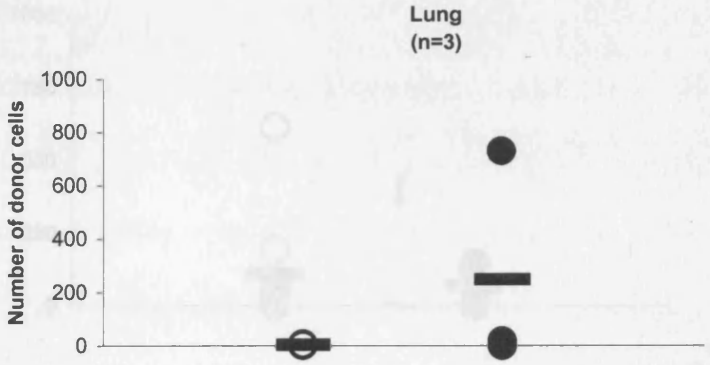
D


MLN
***
2500
2000
1500
1000
500
0
Number of donor cells

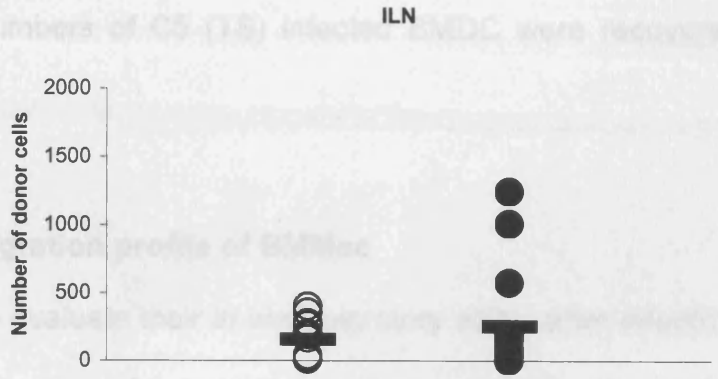
E


Liver
Number of donor cells
2000
1500
1000
500
0

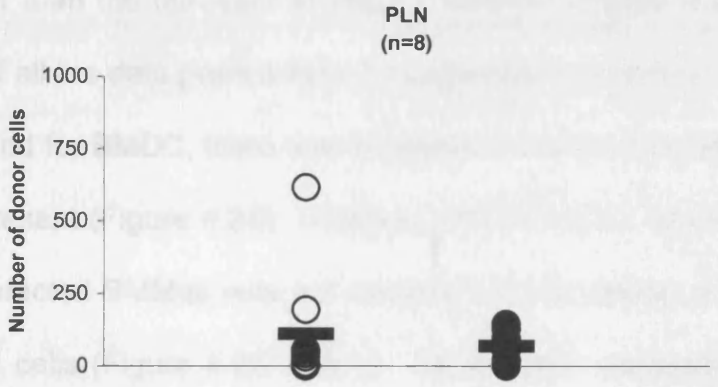
F


Lung
(n=3)
Number of donor cells
1000
800
600
400
200
0

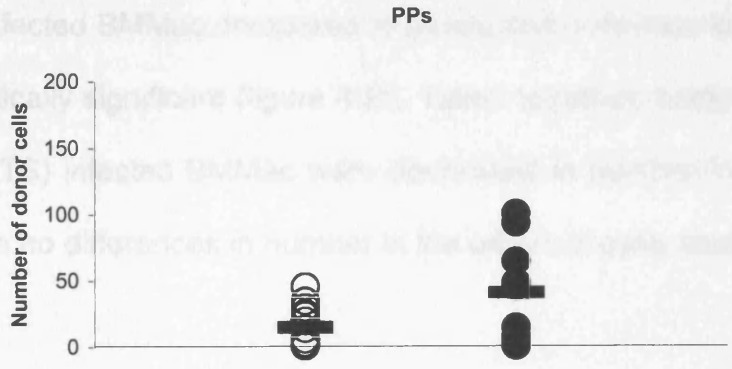
G


ILN
Number of donor cells
2000
1500
1000
500
0

H


PLN
(n=8)
Number of donor cells
1000
750
500
250
0

I


PPs
Number of donor cells
200
150
100
50
0

(PPs) (Figure 4.1G, H and I). Thus, compared to their uninfected counterparts, a decreased number of transferred cells was found in PL, but significantly greater numbers of C5 (TS) infected BMDC were recovered from the spleen and MLN.

4.2.1.2 Migration profile of BMMac

In order to evaluate their *in vivo* migratory ability after infection with C5 (TS), the same procedure of adoptive transfer as BMDC was performed on BMMac. Transferred BMMac could be detected in recipient mice although the numbers were lower than the numbers of BMDC detected (Figure 4.2A). After statistical analysis of all the data pooled from 5 independent experiments, we found that in PL, as found for BMDC, there was a greater number of uninfected than infected BMMac present (Figure 4.2B). However, unlike BMDC, an increased number of C5 (TS) infected BMMac was not observed in the spleen or MLN compared to uninfected cells (Figure 4.2C and D). Comparable numbers of uninfected and C5 (TS) infected cells were also found in the liver and lung (figure 4.2E and F), and ILN and PLN (Figure 4.2G and H). However, a slightly increased number of C5 (TS) infected BMMac compared to uninfected cells was found in PPs, which was statistically significant (figure 4.2I). Taken together, compared to uninfected cells, C5 (TS) infected BMMac were decreased in number in PL but increased in PPs with no differences in number in the others organs analysed.

A

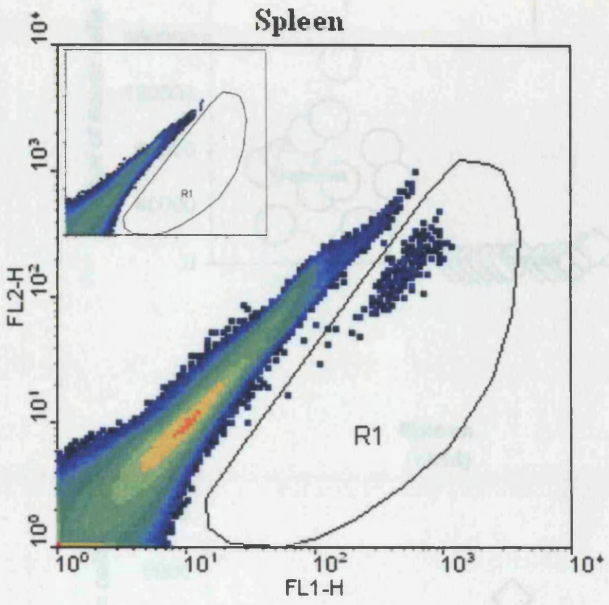
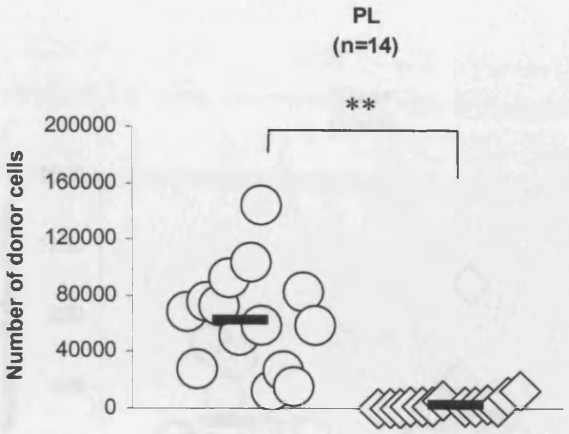


Figure 4.2 *In vivo* migration profile of C5 (TS) infected BMMac compared to uninfected BMMac. Uninfected and 10 MOI C5 (TS) infected BMMac were labelled with CFSE. The labelled BMMac were subsequently adoptively transferred into mice by intraperitoneal injection. Forty-eight hours later, spleen, liver and lung were digested with digesting media containing collagenase (1 mg/ml) and DNase (0.5 mg/ml); lymph nodes (LN) and PPs were mashed with the edges of two frosted slides. Numbers of labelled cells were calculated within 2×10^6 cells collected for each sample by flow cytometry. (A) An example of how the number of labelled cells was derived from the spleen of a recipient mouse. Inset shows the control mouse without transfer, from which the region was set. (B-I) Empty circles (o) indicate uninfected BMMac and empty diamonds (◊) indicate C5 (TS) infected BMMac. Each bar represents the mean value of the number of animals as indicated. Data are pooled from 5 independent experiments and statistic significance was analysed using one-way ANOVA. Number of transferred cells recovered from PL(B), spleen (C) and MLN (D), liver (E) and lung (F), ILN (G), PLN (H) and PPs (I). **, $p < 0.05$.

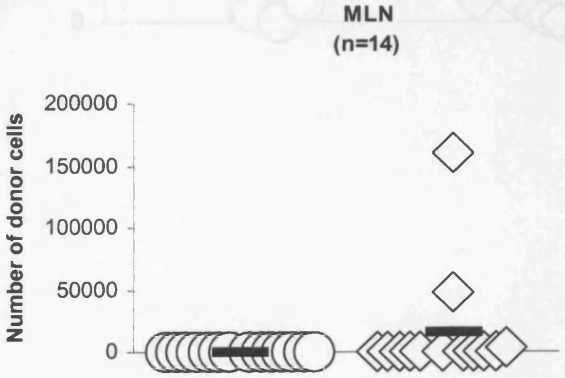
B


PL
(n=14)
**
Number of donor cells
200000
160000
120000
80000
40000
0

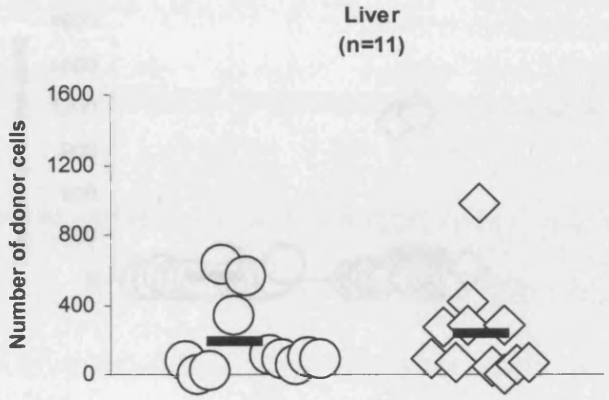
C


Spleen
(n=14)
Number of donor cells
10000
8000
6000
4000
2000
0

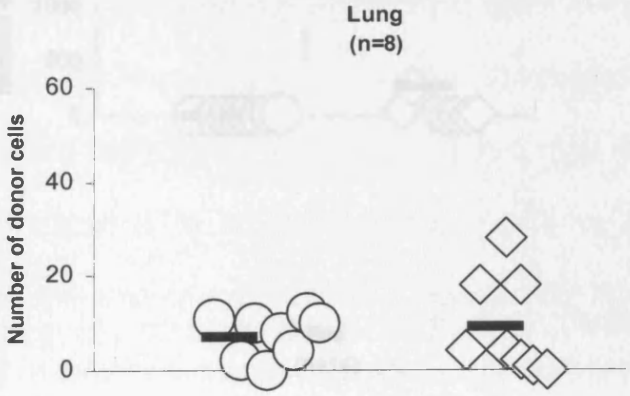
D


MLN
(n=14)
Number of donor cells
200000
150000
100000
50000
0

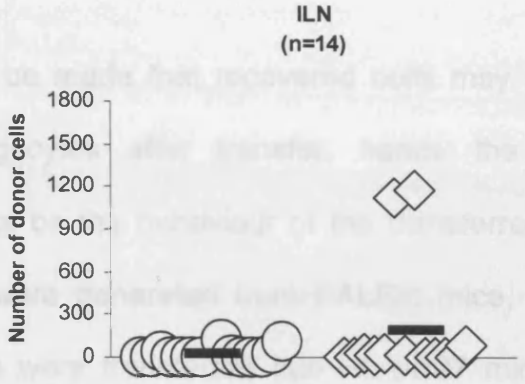
E


Liver
(n=11)
Number of donor cells
1600
1200
800
400
0

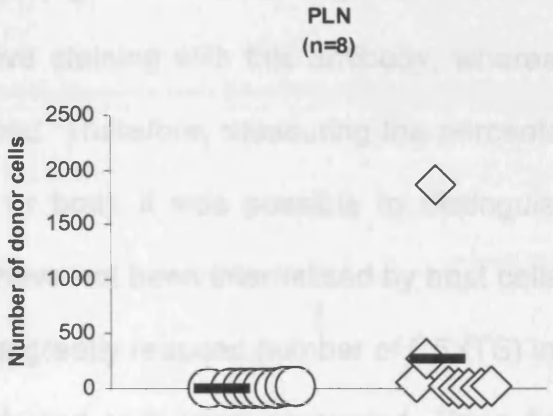
F


Lung
(n=8)
Number of donor cells
60
40
20
0

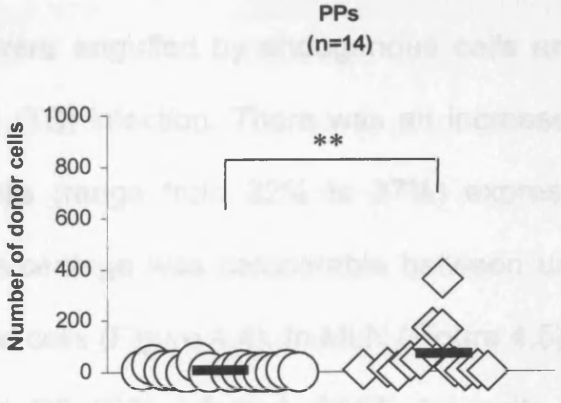
G


ILN
(n=14)
Number of donor cells
1800
1500
1200
900
600
300
0

H


PLN
(n=8)
Number of donor cells
2500
2000
1500
1000
500
0

I


PPs
(n=14)
Number of donor cells
1000
800
600
400
200
0
**

4.2.2 In vivo fate of transferred cells.

An argument can be made that recovered cells may have been taken up by endogenous phagocytes after transfer, hence the migration profiles we observed might not be the behaviour of the transferred cells. To address this question, BMDC were generated from BALB/c mice, and uninfected and C5 (TS) infected cells were transferred into HLA-B27 mice after CFSE labelling. Cells were isolated 48 hours post-transfer from various tissues and stained with an antibody recognising HLA-ABC. Endogenous cells from HLA-B27 host mice would show positive staining with this antibody, whereas the transferred cells would be not stained. Therefore, measuring the percentage of cells positive for CFSE, HLA-ABC or both, it was possible to distinguish between transferred cells that have or have not been internalised by host cells. In PL (Figure 4.3), as shown previously a greatly reduced number of C5 (TS) infected (CFSE+) BMDC compared to uninfected cells were recovered. Upon further analysis of these CFSE (+) cells, less than 15% of uninfected and around 20% of C5 (TS) infected transferred cells expressed HLA-ABC, suggesting that a proportion of transferred cells were engulfed by endogenous cells and that this effect was augmented by C5 (TS) infection. There was an increased proportion of CFSE (+) transferred cells (range from 22% to 37%) expressing HLA-ABC in the spleen and the percentage was comparable between uninfected and C5 (TS) infected transferred cells (Figure 4.4). In MLN (Figure 4.5) up to 70% and 62.6% of uninfected and C5 (TS) infected CFSE (+) cells expressed HLA-ABC. However, it is worthwhile to mention that a very small population of cells was recovered from MLN, which could explain why a higher percentage was

observed. Overall, the data showed that approximately one third of recovered transferred cells represented those engulfed by endogenous cells.

4.2.3 Recovery of bacteria from adoptively transferred cells in tissue.

To assess the role of BMDC in transportation of bacteria, cells were infected *in vitro* with *S. typhimurium* strain SL3261 engineered to express GFP. As the bacteria were labelled with GFP, cells were labelled with the red fluorescent dye CMTMR prior to adoptive transfer. Labelled cells were recovered from various organs at 48 hours post-transfer and the number of labelled cells in those organs calculated. As previously observed *S. typhimurium* infected BMDC could be recovered from spleen and liver and to a lesser extent from MLN and PPs (Figure 4.6 A). In parallel, 2×10^6 cells from spleen and liver, and 2×10^5 cells from MLN and PPs were lysed with Triton X-100, and serial dilutions of the lysate plated on agar plates with and without addition of ampicillin (A+/-). As the GFP plasmid carries an ampicillin-resistant gene, the presence of colonies on ampicillin agar plates would indicate the presence of *S. typhimurium* that had infected the cells *in vitro* and was carried by these cells to the various organs *in vivo*. Bacterial colonies (A+/-) were cultured out, mainly from spleen and liver (Figure 4.6 B) of 2 out of 3 mice receiving GFP expressing SL3261 infected BMDC. The mouse for which no A+ bacteria colonies could be cultured also exhibited very low numbers of recoverable transferred cells in all tissues, indicating an association between the number of recovered CMTMR+ cells and bacterial colonies from all the tissues analysed.

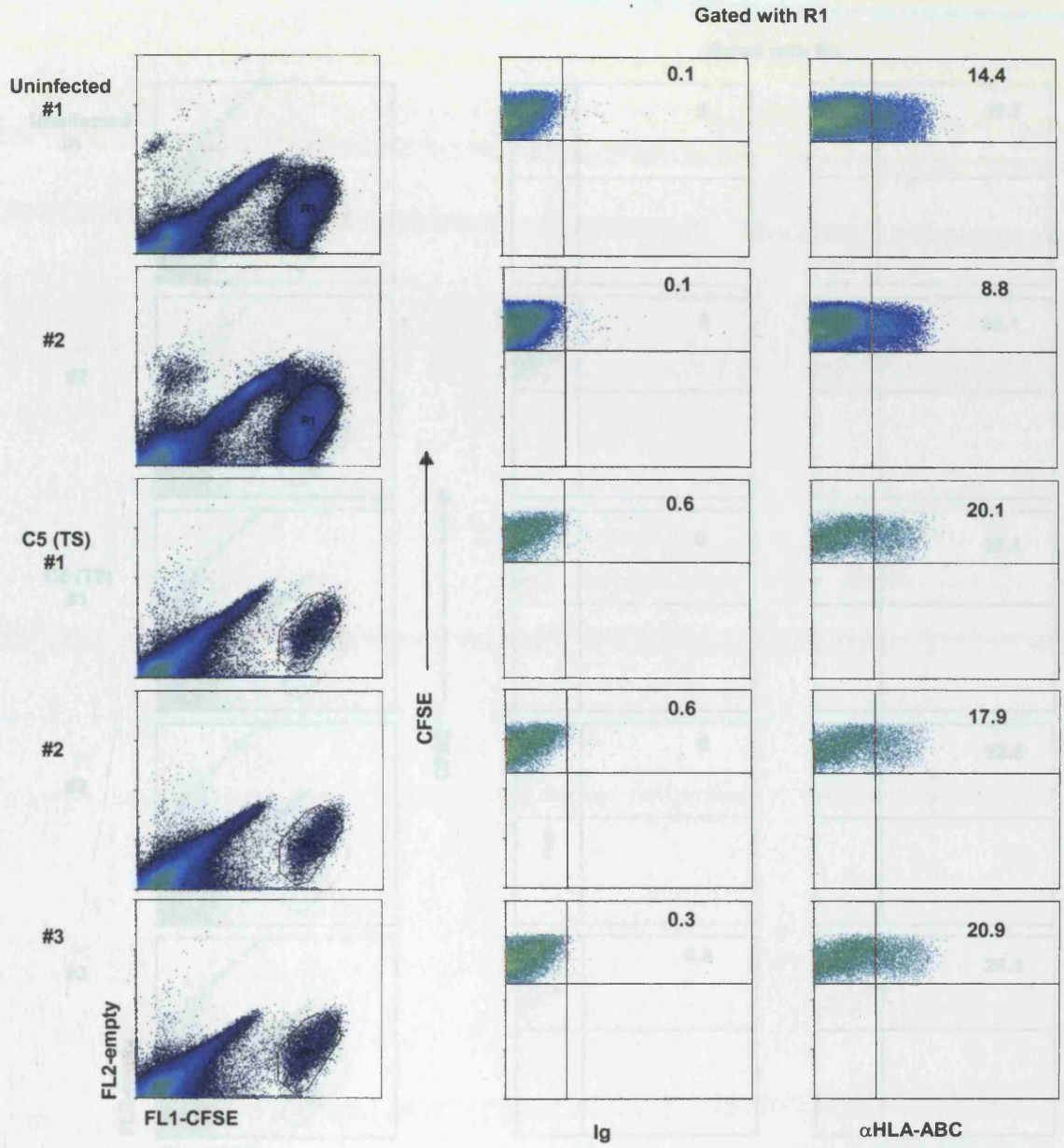


Figure 4.3 Investigation of the fate of adoptively transferred BMDC in peritoneal cavity. DC were generated from bone marrow of BALB/c mice in the presence of GM-CSF. Uninfected and C5 (TS) 10 MOI infected DC were adoptively transferred to HLA-B27/β2m transgenic mice by intraperitoneal injection after labelling with CFSE. Two mice for the uninfected DC transfer group and 3 mice for C5 (TS) were used. Forty-eight hours later, the peritoneal cavity was washed, and cells were harvested from the peritoneal lavage and stained with isotype control antibody (APC conjugate) and antibody recognizing HLA-ABC (APC conjugate). The percentage of cells double positive for CFSE and HLA-ABC is indicated. Results are representative of two independent experiments.

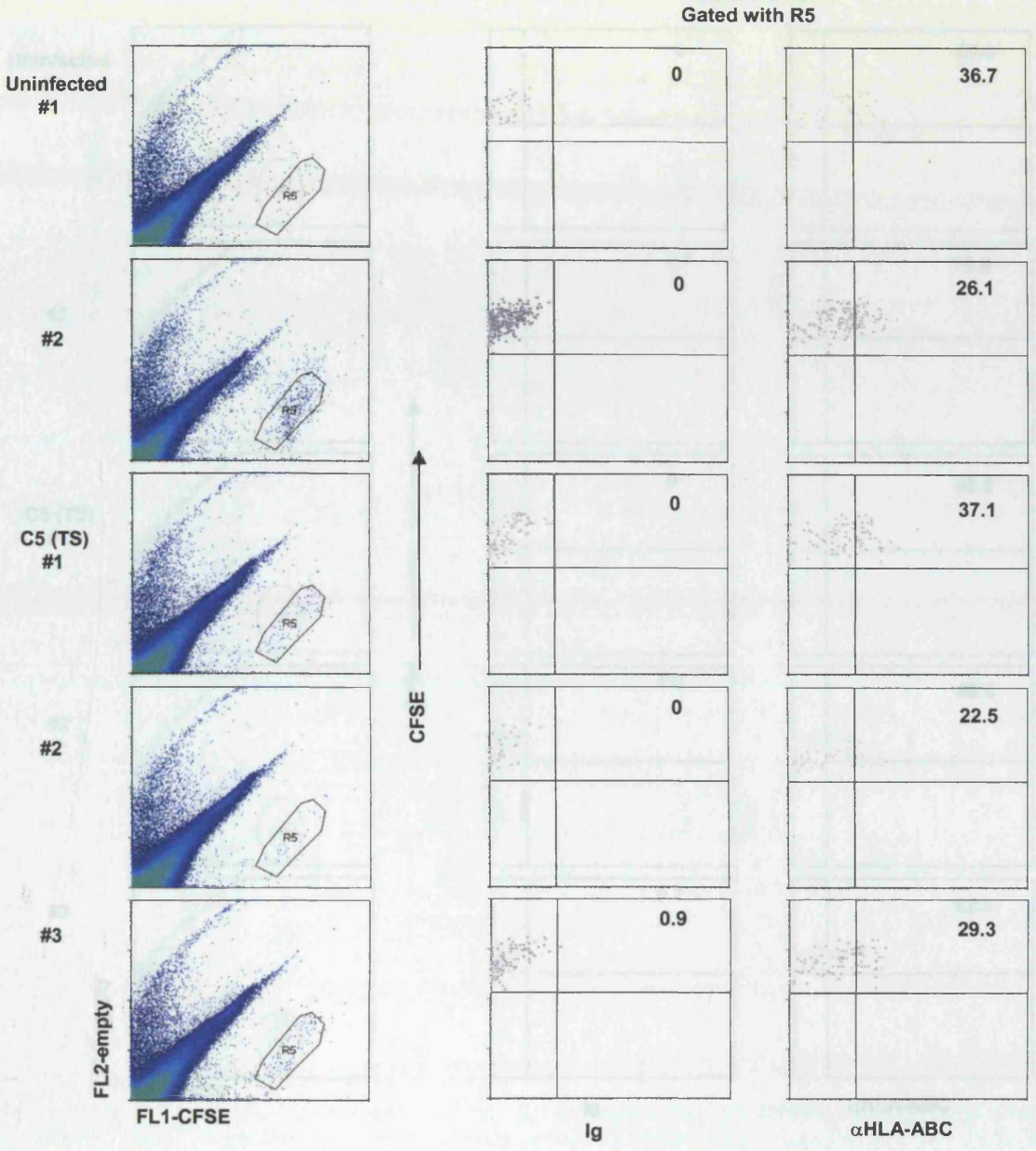


Figure 4.4 Investigation of the fate of adoptively transferred BMDC in spleen. DC were generated from bone marrow of BALB/c mice in the presence of GM-CSF. Uninfected and C5 (TS) 10 MOI infected DC were adoptively transferred to HLA-B27/β2m transgenic mice by intraperitoneal injection after labelling with CFSE. Two mice for the uninfected DC transfer group and 3 mice for C5 (TS) were used. Forty-eight hours later, spleens were digested with collagenase (1 mg/ml) and DNase (0.5 mg/ml), and splenic cells stained with isotype control antibody (APC conjugate) and antibody recognizing HLA-ABC (APC conjugate). The percentage of cells double positive for CFSE and HLA-ABC is indicated. Results are representative of two independent experiments.

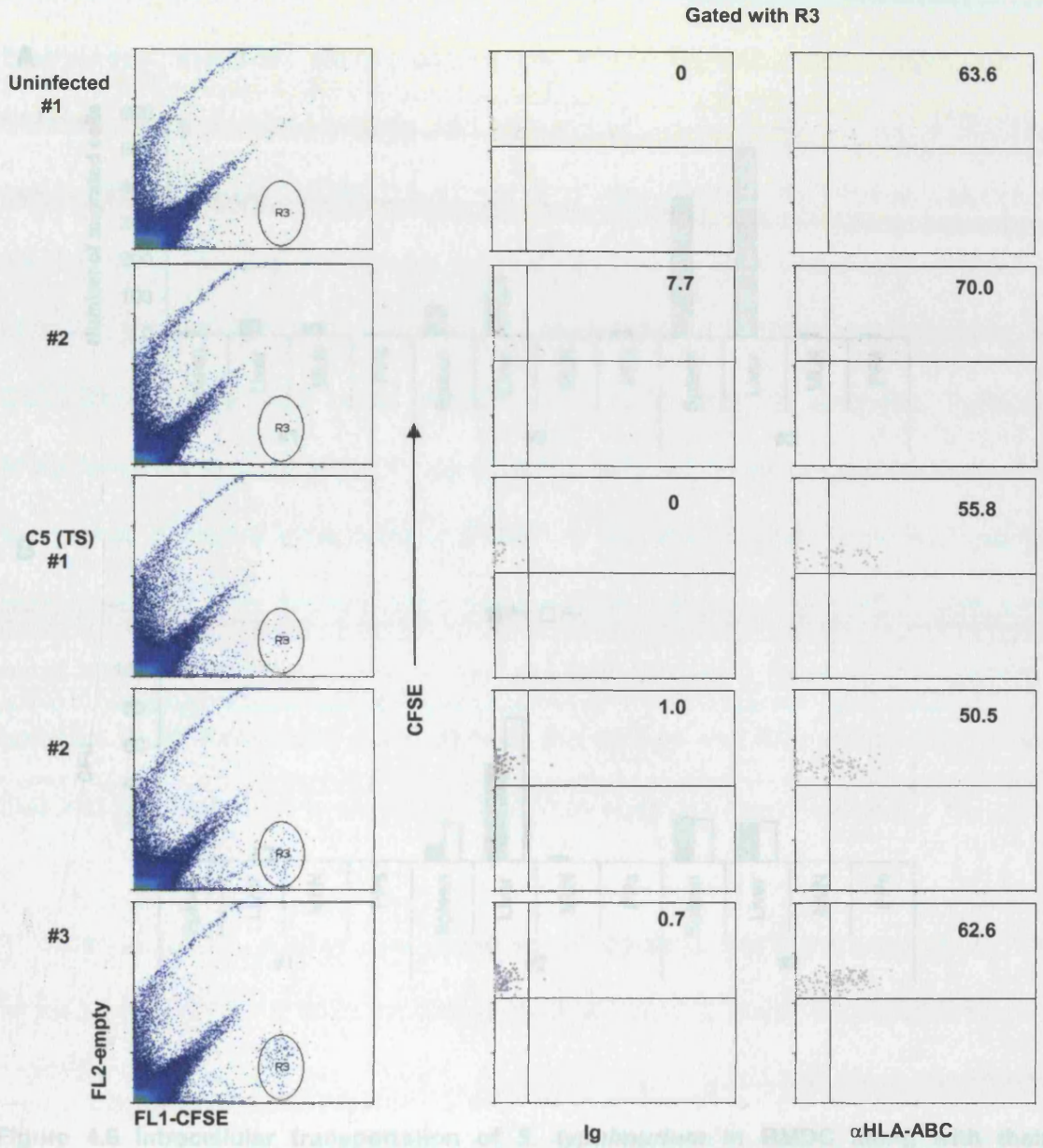


Figure 4.5 Investigation of the fate of adoptively transferred BMDC in mesenteric lymph nodes (MLN). DC were generated from bone marrow of BALB/c mice in the presence of GM-CSF. Uninfected and C5 (TS) 10 MOI infected DC were adoptively transferred to HLA-B27/β2m transgenic mice by intraperitoneal injection after labelling with CFSE. Two mice for the uninfected DC transfer group and 3 mice for C5 (TS) were used. Forty-eight hours later, MLN were mashed and the cells stained with isotype control antibody (APC conjugate) and antibody recognizing HLA-ABC (APC conjugate). The percentage of cells double positive for CFSE and HLA-ABC is indicated. Results are representative of two independent experiments.

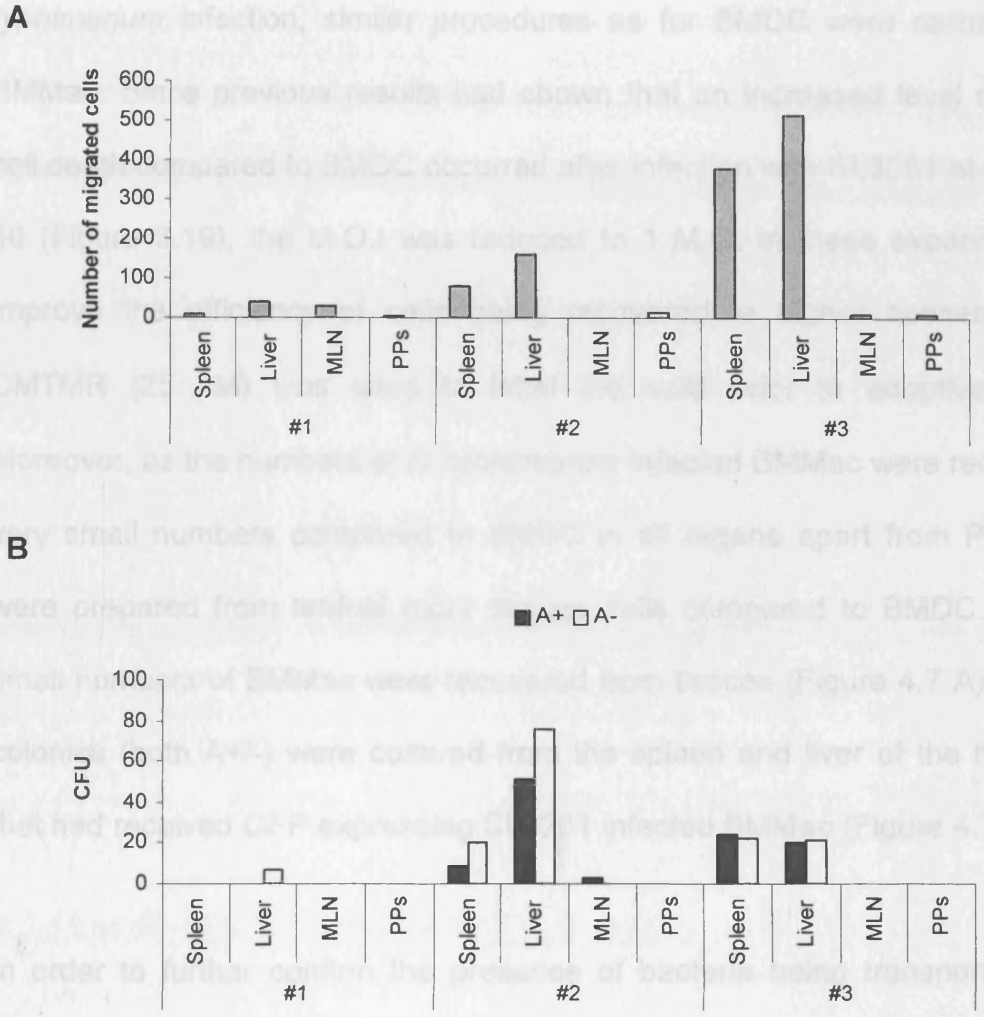


Figure 4.6 Intracellular transportation of *S. typhimurium* in BMDC along with their migration. BMDC were infected with GFP-expressing *S. typhimurium* strain SL3261 at 10 MOI. Twenty-four hours after infection, BMDC were labelled with 5 μM CMTMR and adoptively transferred to HLA-B27/β2m mice (n=3) by intraperitoneal injection. Forty-eight hours later, spleen, liver, mesenteric lymph nodes (MLN) and Peyer's Patches (PPs) were isolated and examined for migrated cells by flow cytometry. The numbers of CMTMR labelled cells within 2×10^6 tissue cells are shown in panel A. In parallel, 2×10^6 cells from spleen or liver or 2×10^5 cells from MLN or PPs were lysed with 0.2% Triton X-100. Serial dilutions of the lysates were plated on agar plates without and with antibiotic (ampicillin indicated as A-/A+) to enumerate the total number of intracellular bacteria. CFU were counted in different tissues as shown in panel B.

In order to evaluate the ability of BMMac to transport bacteria after *S. typhimurium* infection, similar procedures as for BMDC were carried out on BMMac. Since previous results had shown that an increased level of BMMac cell death compared to BMDC occurred after infection with SL3261 at a M.O.I of 10 (Figure 3.19), the M.O.I was reduced to 1 M.O. in these experiments. To improve the efficiency of cells being recovered, a higher concentration of CMTMR (25 μM) was used to label the cells prior to adoptive transfer. Moreover, as the numbers of *S. typhimurium* infected BMMac were recovered in very small numbers compared to BMDC in all organs apart from PL, lysates were prepared from tenfold more tissues cells compared to BMDC. Although small numbers of BMMac were recovered from tissues (Figure 4.7 A), bacterial colonies (both A+/-) were cultured from the spleen and liver of the three mice that had received GFP expressing SL3261 infected BMMac (Figure 4.7 B).

In order to further confirm the presence of bacteria being transported to the tissues by transferred cells, confocal microscopy was used to detect GFP and CMTMR expression in cells. Firstly, BMMac were infected with GFP expressing SL3261 at 1 M.O.I and cells were harvested and labelled with 5 μM CMTMR 24 hours post-infection. Cytospin slides were prepared from these labelled cells and examined by confocal microscopy. The images clearly showed that GFP expressing bacteria (green) were present in CMTMR positive cells (red) (Figure 4.8A top panels). In addition, confocal images revealed that the majority of bacteria were detected as yellow indicating they were intracellular, although a few bacteria were surface bound, as indicated by their green fluorescence in the

confocal image (Figure 4.8A bottom panel). We observed that about 33% of BMMac were infected, which was similar to the previous data (Figure 3.14).

Examination of tissues from mice receiving transferred BMMac after GFP SL3261 infection by confocal microscopy revealed that GFP bacteria were present in spleen (Figure 4.8B). However, although CMTMR labelling of cells was clearly visualised in cytospin preparations (Figure 4.8 A right column), none was detectable in tissues under confocal microscopy.

Taken together, these data suggested that both BMDC and BMMac were able to carry bacteria to the tissues such as the spleen and liver after adoptive transfer.

4.2.4 Localisation of migrated BMDC in spleen

Since CFSE labelling was undetectable under confocal microscopy, in order to determine the localisation of transferred BMDC after migration to the spleen, serial sections were prepared and analysed by immunohistochemistry. An anti-FITC antibody was used to detect CFSE labelled transferred cells and an anti-CD11c antibody as a marker for DC. Transferred cells were distributed mainly around red pulp with some entering the T cells zone of the white pulp (Figure 4.9 left column). As expected, there were a greater number of cells stained with the anti-CD11c antibody compared to the anti-FITC antibody on serial sections due to the presence of endogenous DC (Figure 4.9 right column). However, it was evident that cells staining with both antibodies were co-localised on serial

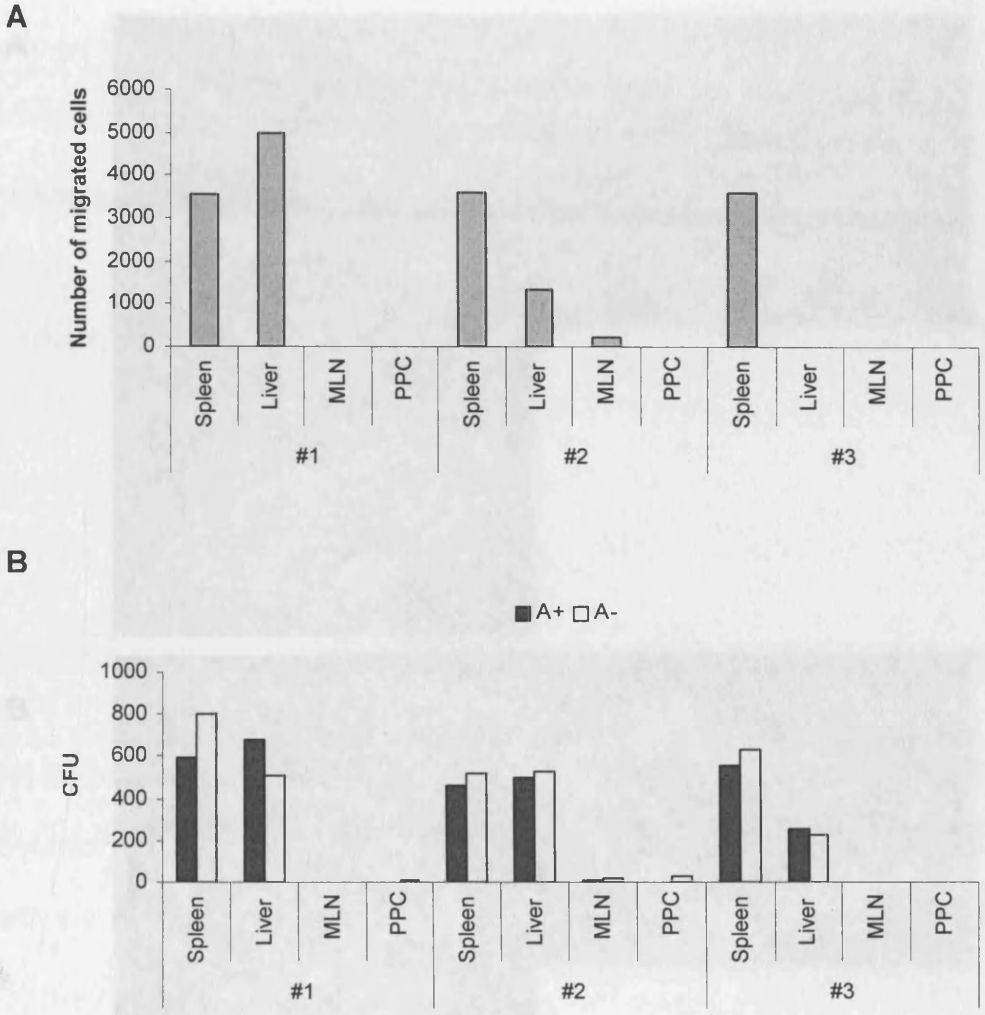


Figure 4.7 Intracellular transportation of *S. typhimurium* in BMMac along with their migration. BMMac were infected with GFP-expressing *S. typhimurium* strain SL3261 at 1 MOI. Twenty-four hours after infection, BMMac were labelled with 25 μM CMTMR and adoptively transferred to HLA-B27/β2m mice (n=3) by intraperitoneal injection. Forty-eight hours later, spleen, liver, mesenteric lymph nodes (MLN) and Peyer's Patches (PPs) were isolated and examined for migrated cells by flow cytometry. The numbers of CMTMR labelled cells within 2×10^6 tissue cells are shown in panel A. In parallel, 2×10^7 cells from spleen or liver or 2×10^6 cells from MLN or PPs were lysed with 0.2% Triton X-100. Serial dilutions of the lysates were plated on agar plates without and with antibiotic (ampicillin indicated as A-/A+) to enumerate the total number of intracellular bacteria. CFU were counted in different tissues as shown in panel B.

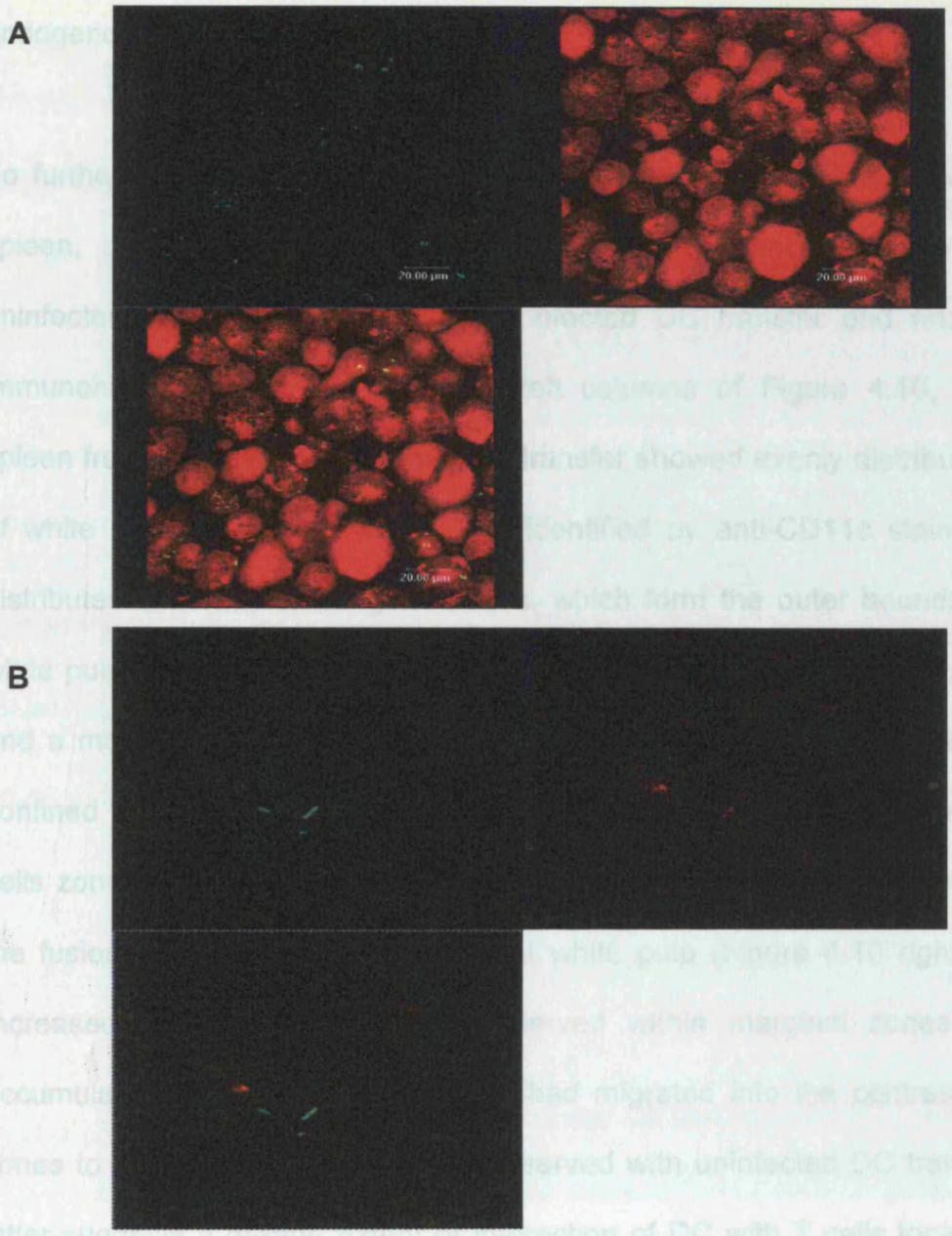


Figure 4.8 Visualisation of GFP-expressing *S. typhimurium in vitro* and *in vivo* by confocal microscopy. (A) BMMac were infected with GFP-expressing *S. typhimurium* strain SL3261 at 1 MOI. The cells were labelled with 5 μM CMTMR. Cytospin slides were prepared from 2×10^5 labelled cells and examined by confocal microscopy. (B) BMMac were adoptively transferred into HLA-B27/β2m mice by intraperitoneal injection after GFP SL3261 infection at 1 MOI and CMTMR labelling. Sections were prepared from spleens isolated from the recipient mice and examined by confocal microscopy after fixation. Intracellular *S. typhimurium* were visualized by GFP fluorescence (green), and CMTMR was detected by red fluorescence. In both A and B, panels show (clockwise from top left) GFP fluorescence, CMTMR fluorescence, and a confocal image of green and red fluorescence. Co-labelling appears yellow in the confocal image.

sections confirming that the distribution of the transferred cells was similar to endogenous DC.

To further examine the influence of BMDC transfer on cell distribution in the spleen, cryostat sections were prepared from the mice with no transfer, uninfected DC transfer and C5 (TS) infected DC transfer and analysed by immunohistochemistry. As shown in left columns of Figure 4.10, a normal spleen from a mouse receiving no cell transfer showed evenly distributed areas of white pulp with similar sizes. DC, identified by anti-CD11c staining, were distributed uniformly in marginal zones, which form the outer boundary of the white pulp. Uninfected DC transfer caused an enlargement of white pulp areas and a marked increase in the number of DC. Although they were still mainly confined to marginal zones, some DC appeared to cluster and to enter the T cells zones (Figure 4.10 middle left). C5 (TS) infected DC transfer resulted in the fusion of greatly enlarged areas of white pulp (Figure 4.10 right bottom). Increased numbers of DC were observed within marginal zones and the accumulated DC formed clusters and had migrated into the centres of T cell zones to a greater extent than that observed with uninfected DC transfer. The latter suggests a greater extent of interaction of DC with T cells localised in T cell zone (Figure 4.10 right bottom).

4.2.5 The ability of transferred cells to migrate to inflamed limbs

The ability of *S. typhimurium* strain C5 (TS) to grow in *vivo* in areas of low temperature makes this bacterium useful for inducing inflammatory arthritis.

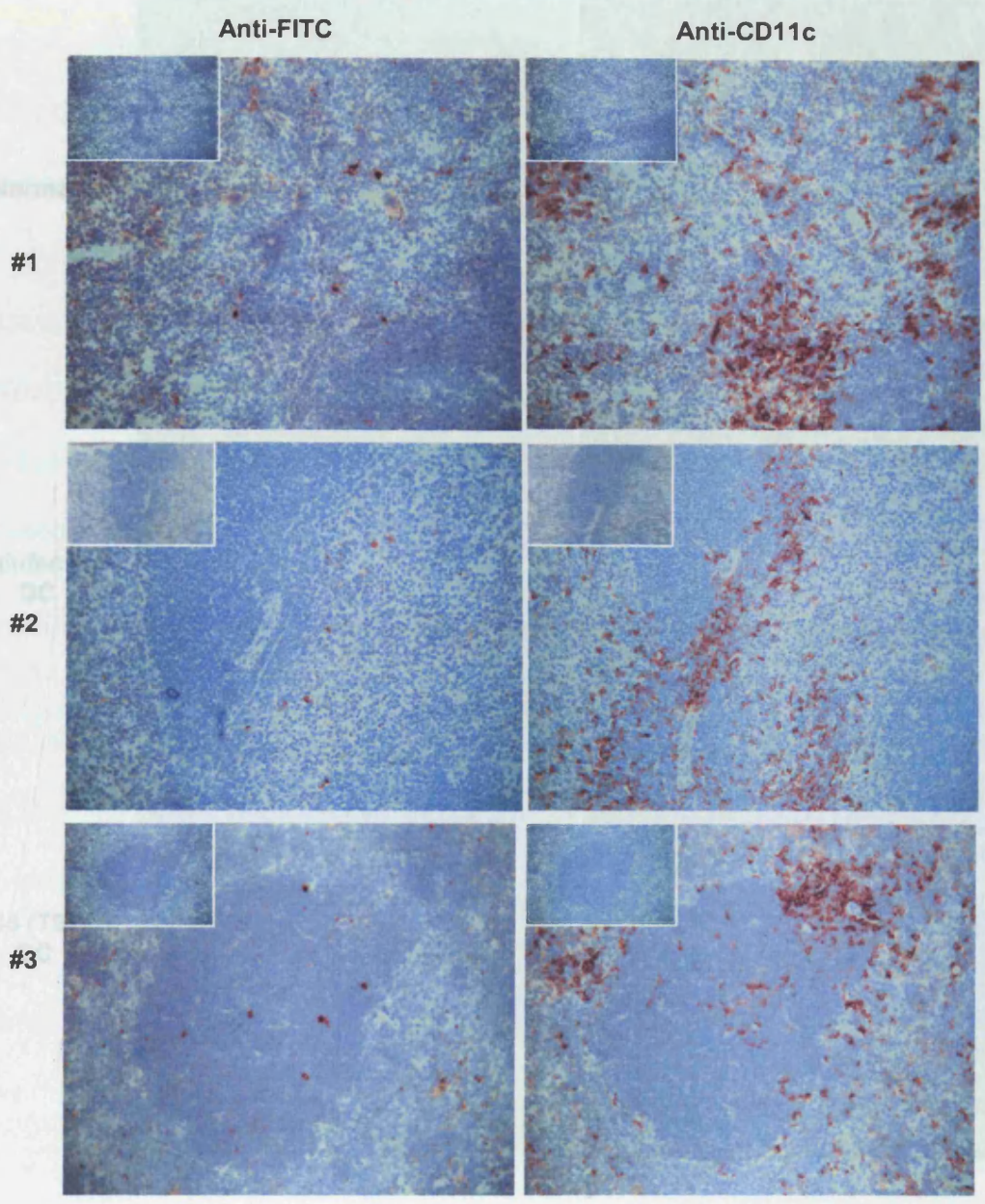


Figure 4.9 Co-localisation of anti-FITC and anti-CD11c staining in spleens of mice injected with CFSE labelled DC. BMDC were infected with C5 (TS) at 10 MOI. Twenty-four hours after infection, uninfected and C5 (TS) infected CFSE-labelled DC were adoptively transferred to HLA-B27/β2m mice. Forty-eight hours later, mice were sacrificed and serial sections were prepared from spleens and examined by immunohistochemistry. Transferred DC were detected with rabbit anti-FITC antibody followed by biotin conjugated anti-rabbit IgG (left panel). Sections in right panels were stained with biotin conjugated anti-CD11c antibody. Sections in all panels were then incubated with Vector ABC complex and the colour reaction (red) was developed by Vector NovaRed kit before counterstaining with hematoxylin. Insets show isotype control staining. Original magnification, × 20 in all panels. Results come from 3 different mice as indicated, and the data are derived from 3 independent experiments.

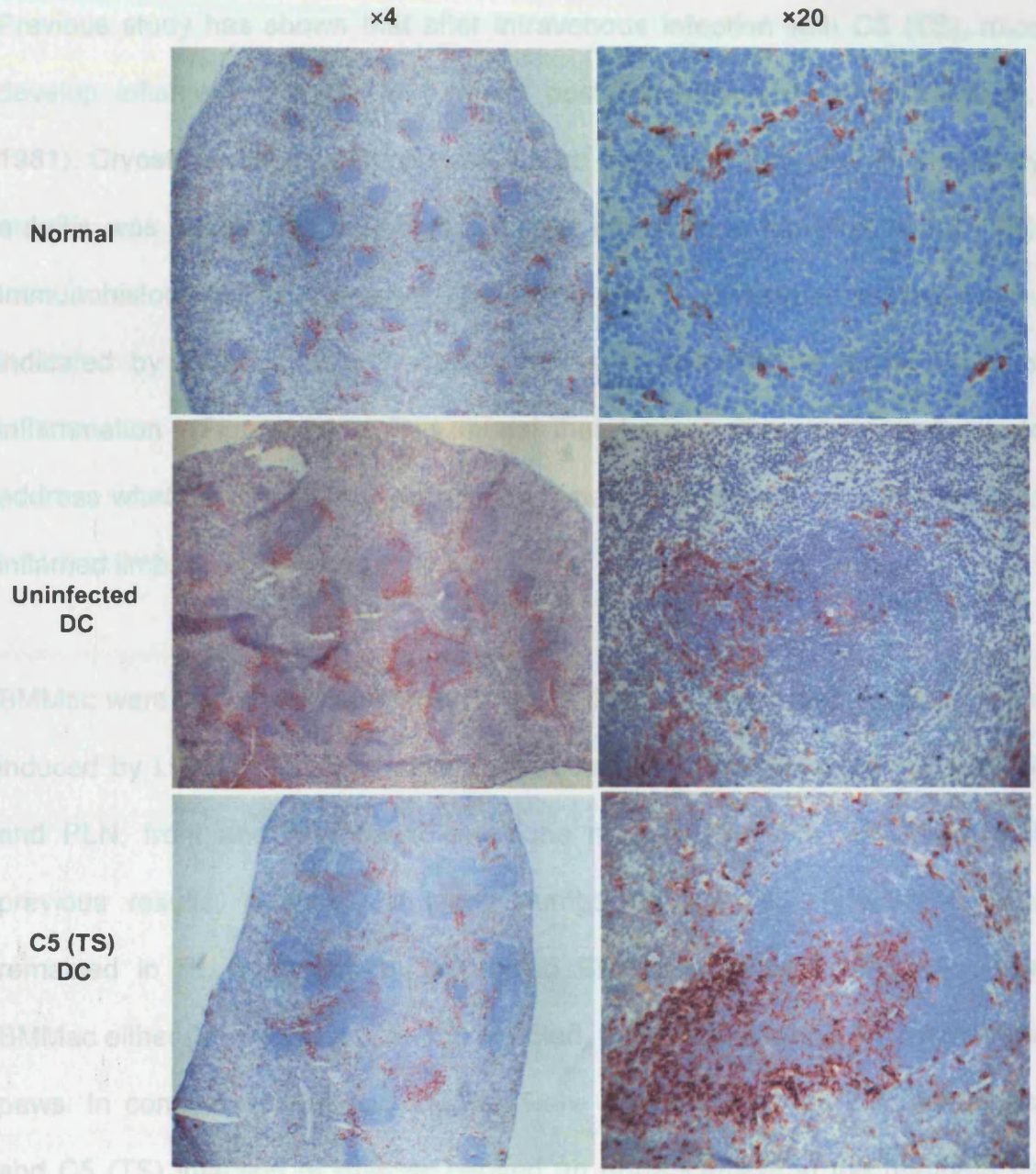


Figure 4.10 Histological changes in the spleen induced by DC transfer. BMDC were infected with C5 (TS) at 10 MOI. Twenty-four hours after infection, uninfected and C5 (TS) infected CFSE-labelled DC were adoptively transferred to HLA-B27/β2m mice. Forty-eight hours later, spleen sections were prepared from normal mice (top panel), mice receiving uninfected DC (middle panel) and mice receiving C5 (TS) infected DC (bottom panel) and examined by immunohistochemical staining. All sections were stained with biotin conjugated anti-CD11c antibody followed by incubation with Vector ABC complex and then the colour reaction (red) was developed by Vector NovaRed kit before counterstaining with hematoxylin. Original magnification, × 4 in left panel and × 20 in right panel. These are representative photomicrographs of histology from 3 experiments.

Previous study has shown that after intravenous infection with C5 (TS), mice develop inflamed joints by 3~4 weeks post inoculation (Hormaeche et al., 1981). Cryostat sections were prepared from inflamed joints after inflammatory arthritis was induced in mice infected with intravenous injection of C5 (TS). Immunohistochemistry revealed that there were numerous macrophages, indicated by F4/80 staining, accumulated and infiltrated in those areas of inflammation (Figure 4.11). This model therefore provides an opportunity to address whether *S. typhimurium* activated macrophages are able to migrate to inflamed limbs after adoptive transfer.

BMMac were adoptively transferred to mice that had developed inflamed joints induced by i.v. C5 (TS), and cells were recovered from PL, axillary LN (ALN) and PLN, front and rear paws and bone marrow (BM). In agreement with previous results, a greatly reduced number of C5 (TS) infected BMMac remained in PL compared to uninfected BMMac (Figure 4.12A). Very few BMMac either uninfected or C5 (TS) infected, migrated into the LNs draining the paws. In contrast, transferred BMMac were isolated from the inflamed paws, and C5 (TS) infection of BMMac caused an enhancement in the numbers we observed. To exclude the possibility that the presence of transferred cells in digested paws were from BM cell contamination, BM alone was also examined and not found to contain any labelled cells. The appearance of the recovered BMMac from paws is highlighted in Figure 4.12B.

To evaluate whether BMDC also have the ability to migrate to inflamed peripheral tissues after transfer, BMDC were injected into mice that had

developed inflammatory arthritis after i.v. C5 (TS) infection. We found that greatly decreased numbers of C5 (TS) infected BMDC compared to uninfected cells were seen in PL (Figure 4.13). Uninfected and C5 (TS) infected transferred BMDC were observed in LNs draining the paws, and the numbers were greater from uninfected than C5 (TS) infected BMDC transfer group, although variation existed among the three mice in both groups. In contrast to the results with BMMac, few if any transferred BMDC were recovered from either front or rear paws (Figure 4.13). These data suggest that BMDC migrate to draining lymph nodes rather than the inflamed limbs.

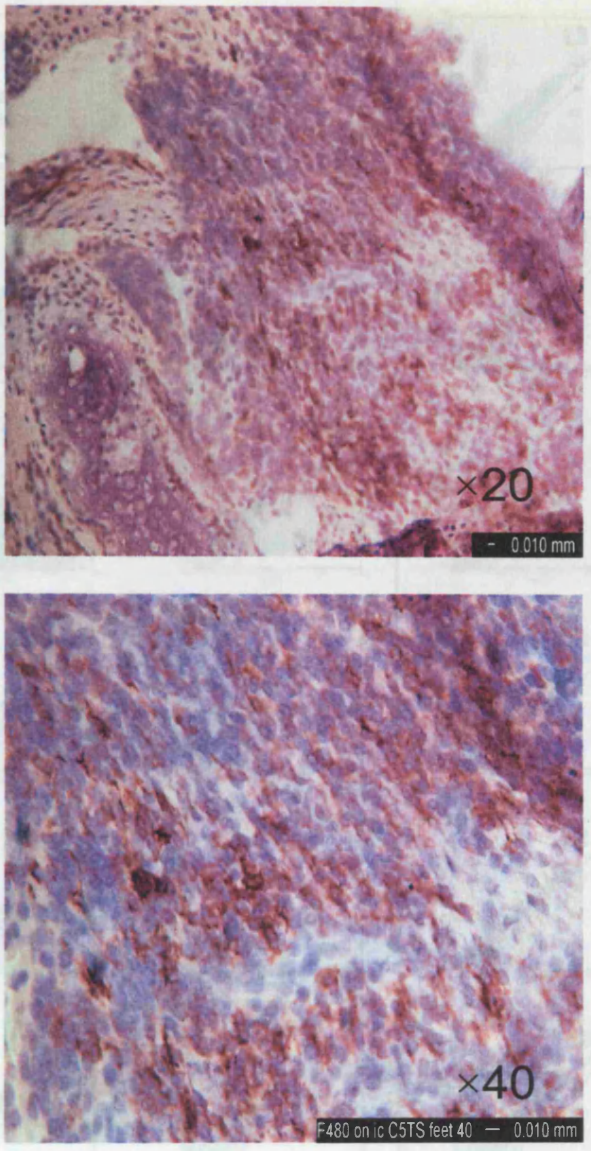


Figure 4.11 Infiltration of macrophages into inflamed tissues in C5 (TS)-induced inflammatory arthritis. HLA-B27/β2m mice were injected with C5 (TS) via the tail vein and mice were monitored for development of inflammatory arthritis. Sections were prepared and F4/80 expression in inflamed joints was examined by immunohistochemistry. Sections were counterstained with hematoxylin.

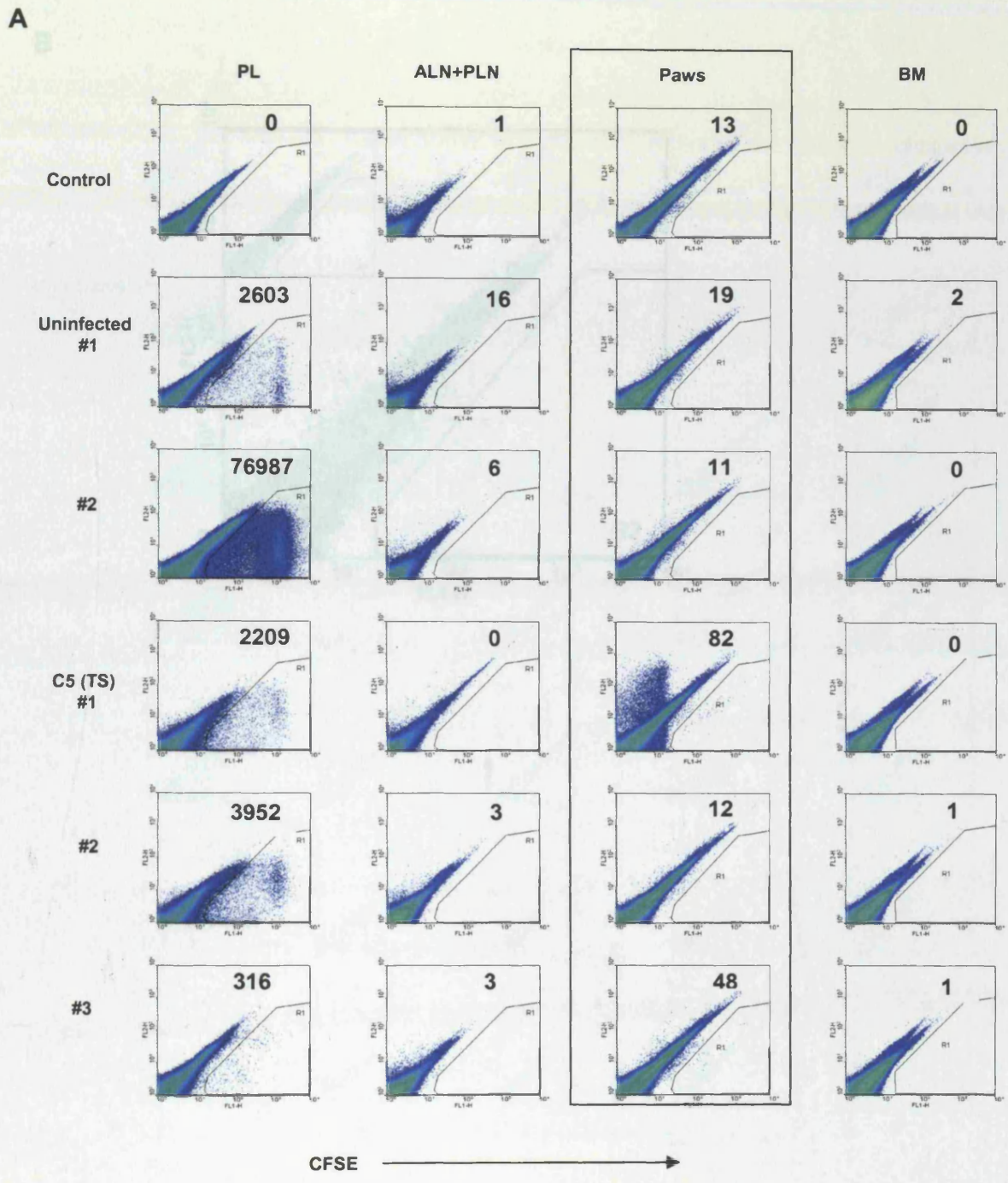
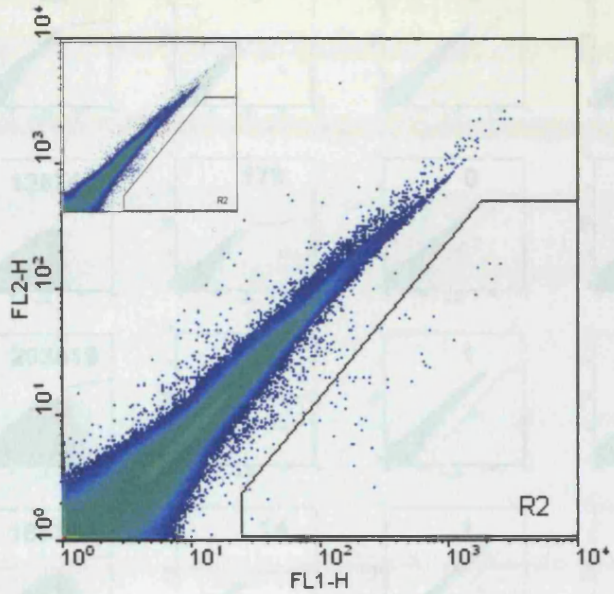


Figure 4.12 Investigation of BMMac migration to inflamed peripheral tissue after C5 (TS) infection. BMMac were infected with C5 (TS) at 10 MOI. Uninfected and C5 (TS) infected BMMac were labelled with CFSE before adoptive transfer to HLA-B27/β2m mice (n=3) by intraperitoneal injection. The recipient mice were previously induced to develop inflamed joints by intravenous injection of C5 (TS). The labelled cells were recovered from tissues as indicated on the top of each column 48 hours post DC transfer. Paws were digested with collagenase (1 mg/ml) and DNase (0.5 mg/ml) for 2 hours and filtered through 40 μm strainers to eliminate cell debris and bones. The number of migrated cells within 2×10^6 tissue cells from each organ is indicated on the right corner of each plot. A mouse without transfer was used as a control. (A) Overall distribution of recovered BMMac in indicated tissues. PL, peritoneal lavage. ALN+PLN, pooled axillary and popliteal LN. BM, bone marrow. (B) Highlighted distribution of transferred cells recovered from paws of a mouse that received C5 (TS) infected BMMac. Inset shows a control mouse without cell transfer, from which the region was set.

B



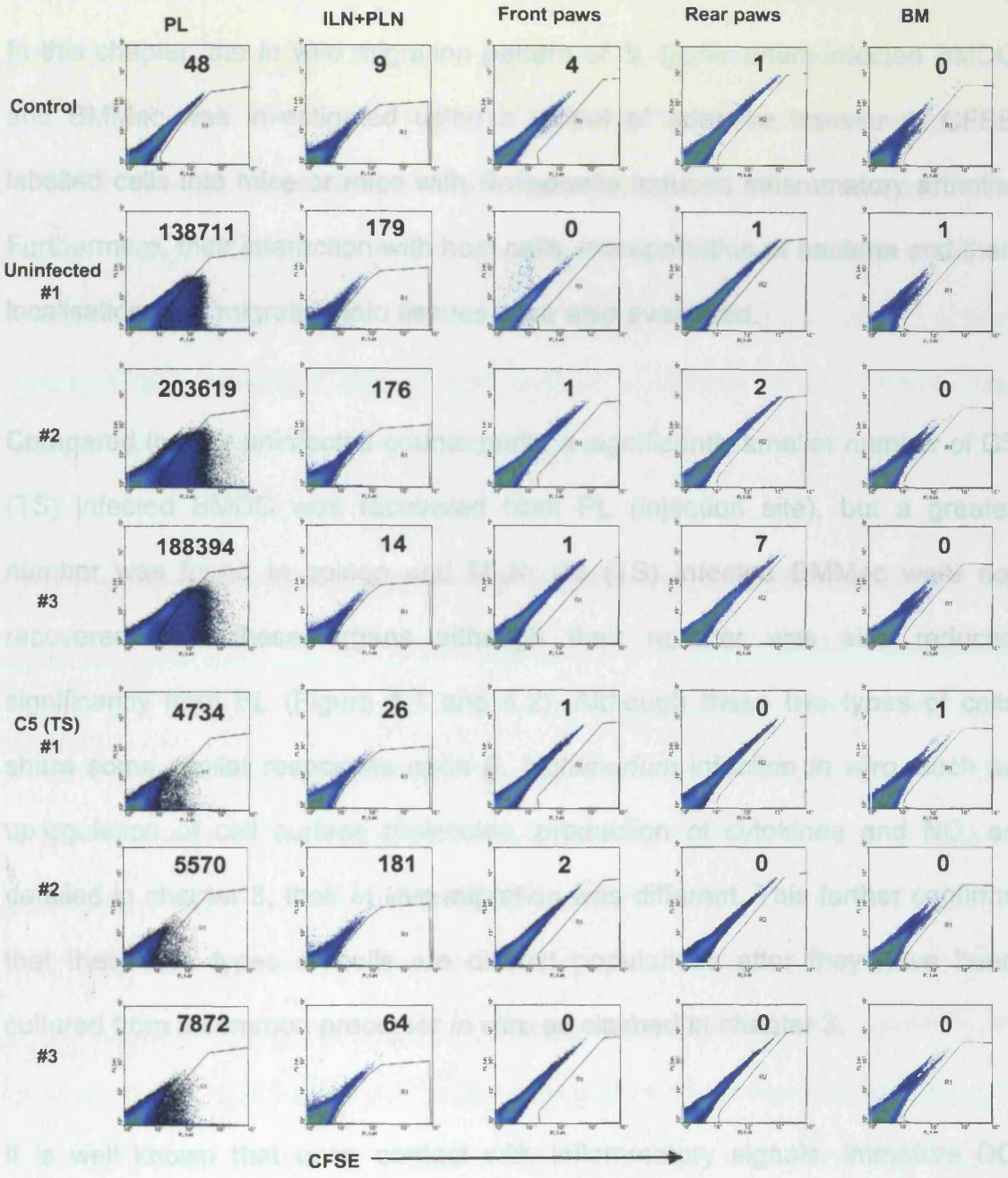


Figure 4.13 Investigation of BMDC migration to inflamed peripheral tissue after C5 (TS) infection. BMDC were infected with C5 (TS) at 10 MOI. Uninfected and C5 (TS) infected DC were labelled with CFSE before adoptive transfer to HLA-B27/β2m mice (n=3) by intraperitoneal injection. The recipient mice were previously induced to develop inflamed joints by intravenous injection of C5 (TS). The labelled cells were recovered from tissues as indicated on the top of graphs 48 hours post DC transfer. Paws were digested with collagenase (1 mg/ml) and DNase (0.5 mg/ml) for 2 hours and filtered through 40 μm strainers to eliminate cell debris and bones. The number of migrated cells within 2×10^6 tissue cells from each organ is indicated on the right corner of each plot. A mouse without transfer was used as a control. PL, peritoneal lavage. ILN+PLN, pooled inguinal and popliteal LN. BM, bone marrow.

4.3 Discussion

In this chapter, the *in vivo* migration pattern of *S. typhimurium*-infected BMDC and BMMac was investigated using a model of adoptive transfer of CFSE labelled cells into mice or mice with *Salmonella* induced inflammatory arthritis. Furthermore, their interaction with host cells, transportation of bacteria and their localisation after migration into tissues were also evaluated.

Compared to their uninfected counterparts, a significantly smaller number of C5 (TS) infected BMDC was recovered from PL (injection site), but a greater number was found in spleen and MLN. C5 (TS) infected BMMac were not recovered from these organs although their number was also reduced significantly from PL (Figure 4.1 and 4.2). Although these two types of cells share some similar responses upon *S. typhimurium* infection *in vitro*, such as upregulation of cell surface molecules, production of cytokines and NO, as detailed in chapter 3, their *in vivo* migration was different. This further confirms that these two types of cells are distinct populations after they have been cultured from a common precursor *in vitro* as claimed in chapter 3.

It is well known that upon contact with inflammatory signals, immature DC undergo a maturation process and migrate from the periphery to the T cell area of LN, where they launch the adaptive immune response (Banchereau et al., 2000). Although using a different agent (irradiated mycobacterium tuberculosis) to activate cells *in vitro*, it has been reported recently that DC but not macrophages migrate to the draining LN after co-transfer (Bhatt et al., 2004). In addition, these transferred DC were able to initiate a local primary Th1

response, indicated by IFNγ secretion by antigen specific T cells. Macrophages failed to do so due to their failure to migrate to draining LNs (Bhatt et al., 2004). Macrophages and DC, both phagocytic cells, are able to capture antigen and induce inflammatory response in the periphery. However, macrophages have a greater ability than DC to engulf bacteria *in vitro,* manifested by a higher infection rate and increased cytokine production. DC are more sensitive than macrophages to danger signals and migrate efficiently to SLO where they present antigen to T cells.

The role of macrophages in antigen presentation is more ambiguous. In our experiments, there were no more antigen stimulated cells than uninfected cells migrating to SLO. What roles do macrophages play at this stage? It has been observed that subcutaneous challenge with outer membrane protein A (OmpA) of gram-negative bacteria induces an enlargement of draining LN with a higher proportion of DC and macrophages (Jeannin et al., 2003); Adoptive transfer of OmpA pulsed DC and macrophages into footpads demonstrated that the higher proportion of DC in draining LN was due to their migration from periphery. In contrast, it was concluded, due to the absence of transferred macrophages in draining LN, that the higher proportion of macrophages in draining LN occurred mainly from recruitment of peripheral blood macrophage precursors (Jeannin et al., 2003). Taken together with their greater production of inflammatory cytokines in response to *S. typhimurium* infection, these data suggest that macrophages function primarily to control bacterial replication through innate immunity and recruitment of more phagocytic cells rather than by initiating specific adaptive immunity.

Surprisingly, significantly greater numbers of *S. typhimurium* infected macrophages compared to uninfected cells were recovered in PPs (Figure 4.2 I). For this reason, we tested whether macrophages showed enhanced expression of lymphocyte Peyer's patch adhesion molecule (LPAM-1, also named $\alpha4:\beta7$) after *S. typhimurium* infection. This molecule is normally expressed by a subset of T cells and contributes to their homing to mucosal endothelia including PPs (Mora et al., 2003). Expression of integrin $\alpha4$ (no β subunit antibody available) was tested on BMMac and BMDC with and without *S. typhimurium* infection. We found that it was constantly expressed on both types of cells; *S. typhimurium* infection did not increase its expression on either BMMac or BMDC (data not shown).

It has been previously shown that DC but not macrophages are able to migrate to draining LNs *in vivo* after being activated by various stimuli *in vitro* (Bhatt et al., 2004; Jeannin et al., 2003). Therefore, the migration patterns we observed after *S. typhimurium* infection followed the general migration pattern of these two types of cells upon encountering microbes or microbial products.

In order to establish whether the transferred cells had been taken up by endogenous cells, BMDC were propagated from BALB/c bone marrow cells and transferred into HLA-B27 mice. If HLA-B27 negative transferred cells were engulfed by HLA-B27 expressing endogenous cells, a CFSE and HLA-B27 double positive population would be detected. An increasing proportion of transferred cells from PL (<20%), spleen (<40%) to MLN (<70%) had been engulfed by host cells (Figure 4.3-4.5). Therefore, it is possible that the

migration profiles we observed for transferred DC and macrophages after *S. typhimurium* infection might not be the cells we originally transferred. However, the percentage of engulfed cells in the PL (CFSE+ & HLA-ABC+) was less than 15 or 20% for uninfected and *S. typhimurium* infected DC respectively, indicating that after two days *in vivo*, only a small proportion of cells were engulfed in PL. The slightly increased percentage of the scale of infected versus uninfected DC that was internalised might suggest that transferred *S. typhimurium* infected DC provided an increased "danger signal" to the host than uninfected cells.

The higher proportion of engulfed cell population observed in spleen and MLN could be partly artefactual, related to the fact that relatively few CFSE+ cells were detected in these organs. Thus, the presence of a small number of autofluorescent host cells in the CFSE+ gate would make the apparent population of CFSE+ HLA-B27+ cells seem bigger than it actual was. A larger number of cells would need to be acquired to address this. For transferred cells that were taken up by host cells, there are two possibilities to account for their localisation in spleen and MLN. One is that cells were taken up firstly by endogenous cells in the PL, which then migrated to the spleen and MLN. Another is that injected cells migrated firstly to spleen and MLN and then were engulfed by host cells in these organs. If the former possibility were true, we would have expected to find a similar pattern of migration for DC and macrophages, rather than the differential migration patterns observed. Therefore, our overall data support the latter explanation.

Since all cells have a limited life span (Opferman and Korsmeyer, 2003), cells transferred into mice must undergo eventual cell death and will be cleared by host cells through phagocytosis. After acting with T cells, migrating DC are thought to die within the LN, because they can be detected in afferent lymph that access the T-cell areas, but not in efferent lymph (Banchereau and Steinman, 1998; Steinman, 1991). It has also been suggested that CTL-mediated killing of DC is important to avoid unnecessary further presentation of antigen and to eliminate infected cells themselves; this mechanism is particularly important for the host to clear virus infection (Ronchese and Hermans, 2001). Therefore, the increased proportion of engulfed, transferred DC in SLO may reflect the physiological clearance of these cells.

To further clarify that the migration pattern observed was the behaviour of transferred cells, fixation of cells prior to transfer, as has been attempted in other studies (Ibrahim et al., 1992; Rosen and Gordon, 1990), may prove useful. In this case, for example, active migration of transferred DC would be abolished and they should not be found in SLO. However, if transferred *S. typhimurium* infected DC could still be recovered from SLO, this would indicate that endogenous cells were responsible for transporting CFSE+ cells after phagocytosis.

Transportation of *S. typhimurium* by BMDC and BMMac was also examined. In order to visualise bacteria by confocal microscopy and also to identify the presence of transported bacteria recovered from tissues, *S. typhimurium* strain SL3261 engineered to express GFP was utilised in these experiments. This

strain contained an ampicillin resistant gene, which allowed us to identify them by culturing on ampicillin containing agar plates. The results shown in Figure 4.6 and 4.7 revealed that *S. typhimurium* was carried to tissues by both BMDC and BMMac. Although a different strain of *S. typhimurium* was used in these experiments, a similar pattern of migration to that observed with C5 (TS) infected cells was seen. Notably, though the recovery of CMTMR labelled BMDC seemed lower than BMMac (tenfold lower) (panel A of Figure 4.6 and 4.7). This was potentially due to the use of a lower concentration of CMTMR for DC (5 μM vs 25 μM), which could have led to some recovered cells not being visualised by flow cytometry. The similar efficiency of bacterial recovery (panel B of Figure 4.6 and 4.7) may be related to their similar infection rate after *in vitro* infection. However, it should be noted that 10 times more tissue cells were used for measuring CFU for macrophages than DC (legend of Figure 4.6 and 4.7).

The presence of GFP expressing *S. typhimurium* in the spleen was confirmed by confocal imaging (Figure 4.8 panel B). Although we have successfully shown the colocalisation of GFP bacteria (green) and CMTMR labelled cells (red) on cytospin slides after *in vitro* infection (Figure 4.8 panel A), we failed to visualise the red fluorescence of CMTMR in tissues (Figure 4.8 panel B); this was also true for CFSE labelling. Therefore, we chose to show the presence of CFSE labelled cells *in vivo* by using an anti-FITC antibody and non-immunofluorescent detection rather than direct confocal analysis of the CFSE. Although *S. typhimurium* infected macrophages did not show an enhanced ability to migrate to the spleen and MLN, a small number of cells were constantly found in these tissues.

The interaction of *S. typhimurium* with APCs including DC and macrophages has been extensively studied *in vitro* (Chakravortty et al., 2002; Garvis et al., 2001; Svensson et al., 1997b; Yrlid et al., 2001a), but little direct analysis of these interactions *in vivo* has been reported. Using combined confocal laser scanning microscopy and computerised image analysis techniques with immunostained sections of liver, Richter-Dahlfors and colleagues have shown that *S. typhimurium* resides intracellularly in macrophages of mouse liver when the mouse is infected intravenously with only a small number of bacteria, conditions they claim mimic a natural infection (Richter-Dahlfors et al., 1997). In another study, *S. typhimurium* was shown to be transported from the gastrointestinal tract to the bloodstream by CD18-expressing phagocytes, possibly consisting of DC and/or tissue macrophages as early as 15 minutes after oral inoculation (Vazquez-Torres et al., 1999). More recently, DC have been shown to express tight junction proteins and penetrate gut epithelial monolayers to access the lumen and directly sample bacteria *in vivo;* this was demonstrated by infection the ligated loop of the small intestine in mice with GFP-expressing *S. typhimurium* (Rescigno et al., 2001).

Our results show that adoptively transferred infected DC and macrophages were able to transport intracellular *S. typhimurium,* acquired from *in vitro* infection, to two of the major organs involved in the systemic infection (spleen and liver). Together with the published observations discussed above, these results suggest that these cells may be used by *S. typhimurium* for the spread of this intracellular pathogen throughout the body. Unlike BMDC, we did not find

our transferred *S. typhimurium* infected macrophages in large numbers in spleen and draining LNs. However, they were able to reach all the tissues we examined in small numbers. In infected macrophages, *S. typhimurium* has evolved a mechanism that depends on the activity of its SPI-2 type three secretion system to shield itself from cytosolic anti-*Salmonella* activity and thereby to survive and replicate itself in a modified vacuole (Linehan and Holden, 2003); Furthermore, enzymes and products synthesised by *S. typhimurium* efficiently reduce the effectiveness of bacteriocidal and bacteriostatic free radicals (such as NO) generated by macrophages (Linehan and Holden, 2003). Together with their ability to access various tissues after adoptive transfer, macrophages are likely to be used by *S. typhimurium* as a vehicle to facilitate their systemic spreading.

The histological structure of the spleen is divided into red pulp and white pulp, with marginal zone encircling the white pulp. The complex architecture of the spleen facilitates close contact between blood-borne antigens and immune cells, which are segregated according to their functions into distinct splenic compartments (Yadava et al., 1996). Circulating antigens are initially screened in the marginal zone, in which extremely potent phagocytic cells, the marginal zone macrophages, are present and can phagocytose large foreign particles, such as bacteria and effete red blood cells. Further filtration of the blood takes place in the filtration beds of the red pulp, where the majority of endogenous DC normally reside and can further screen blood borne antigens. After taking up antigen, DC migrate to the outer area of white pulp, in which they present antigen to T cells and lead to clustering and enrichment of antigen-specific T

cells. B and T cells recirculate also through the outer area of white pulp on their way to follicles and the inner area of the white pulp respectively, which facilitates the DC antigen presentation to T cells (Kraal, 1992).

We have demonstrated by immunohistochemistry that transferred DC mainly migrated into red pulp and marginal zone areas. Some cells, especially *S. typhimurium* infected DC, also appeared interspersed around the white pulp. Staining of serial spleen sections with various antibodies revealed that anti-FITC staining (indicating transferred DC) was co-localised with the distribution of endogenous CD11c expressing cells (Figure 4.9).

In the spleen, CD11c + DC can be further divided into three subsets: CD8α+, CD8α-CD4+, and CD8α-CD4- (Kirby et al., 2001; Moser and Murphy, 2000). Subset-specific responses of splenic DC to *Salmonella* infection have been documented (Kirby et al., 2001). In that study, CD4+ DC were shown to alter their localisation from the white pulp borders and margins of the B cell areas to the centre of B cell area with their numbers remaining constant post-*Salmonella* infection. Conversely, the red pulp CD8+ and CD8-CD4- DC dramatically increased in number concomitant with redistribution within the red pulp as an early response, followed by migration to T cells area later during *Salmonella* infection (Kirby et al., 2001).

Our data showed that CD11c staining in both the red pulp and the white pulp of spleen sections appeared more frequent than could be accounted for by coincident anti-FITC+ staining. This suggested that both transferred and

endogenous DC were able to migrate to T cell area to present antigens upon *Salmonella* infection. The antigens acquired by these cells may differ, as endogenous DC could take up the dead transferred cells or engulf bacteria released by dead cells. Migration of transferred DC from the red pulp to white pulp by 24 hours after intravenous or subcutaneous injection has been previously reported (Austyn et al., 1988). In addition, data from more recent studies have confirmed that endogenous CD11c+ DC also migrate from the marginal zone of the spleen into the CD4+ T cell area after they sense the presence of antigens from the blood stream (Leisewitz et al., 2004; Sher and Reis e Sousa, 1998). In contrast, Leisewitz and colleagues showed that during infection, macrophages remain confined to the red pulp area and B cells show no appreciable change in distribution (Leisewitz et al., 2004).

It has been shown that BMDC and BMMac are efficient at processing and presenting bacterial antigens from *S. typhimurium* infections, although the pathways they use to accomplish this are not identical (Yrlid et al., 2000). Both CD8α+ and CD8α- splenic DC are able to process live bacteria for peptide presentation on MHC-I and MHC-II *in vitro* (Yrlid and Wick, 2002). Furthermore, splenic DC isolated from *S. typhimurium* infected mice could present bacterial antigens upon co-culture with specific T cells directly *ex vivo* (Yrlid and Wick, 2002). *S. typhimurium* can induce apoptosis of infected macrophages (Chen et al., 1996a; Chen et al., 1996b; Monack et al., 1996). Interestingly, an alternative antigen presentation pathway whereby bystander DC present antigens derived from macrophages induced to undergo apoptosis by *S. typhimurium* also has

been confirmed (Yrlid and Wick, 2000). Therefore, antigen presentation occurring in natural *Salmonella* infections could be complex.

Compared to the normal spleen, disruption of normal splenic architecture was noticeable after DC transfer (Figure 4.10). The latter was manifested as the enlargement and fusion of white pulp areas, presumably due to the expansion of clones of T cells and/or immigration of transferred and endogenous DC. In comparison to the small number of DC distributed along the marginal zone and red pulp in a normal spleen, a large number of CD11c+ cells was observed after transfer of *S. typhimurium* infected DC; this was also observed to a lesser extent in the spleen of mice receiving uninfected DC. Since relatively small number of transferred DC (even *Salmonella* infected) were detected (Figure 4.9, left column), recruitment of CD11c+ cells from blood-borne cells likely occurred. Such a mechanism has been suggested in a previous study showing that splenic DC numbers increase after administration of soluble *Toxoplasma gondii* extract, which was assumed to result from an influx of blood-borne cells (Reis e Sousa et al., 1997). Thus, the increase in CD11c+ cell number is a consequence of the migration of transferred DC endogenous DC to the spleen. These findings are consistent with a previous study showing that the absolute number of CD8α+ and CD8α-CD4- DC, but not CD4+ DC are significantly increased in the spleen after oral *Salmonella* infection (Kirby et al., 2001).

Previous reports suggest that monocytes play a role in transporting *Salmonella* antigens to the joints of patients with ReA (Kirveskari et al., 1998), since in our model, it was not possible to obtain large numbers of monocytes to do the

adoptive transfer experiment, bone marrrow derived macrophages and DC were used. To examine the ability of BMDC and BMMac to traffick to inflamed joints, CFSE labelled cells were injected i.p. into mice that had received an i.v. injection of C5 (TS) 3 weeks previously; the latter has been shown to induce inflammatory arthritis efficiently in mouse (Hormaeche et al., 1981). In our study, the large number of F4/80+ macrophages infiltrating into inflamed tissue (Figure 4.11) indicated that the inflammatory arthritis induced by i.v C5 (TS) served as a surrogate model of ReA with augmented inflammation in affected joints. The number of transferred cells in various tissues was examined using flow cytometry 48 hours post-transfer. A small number of BMDC were recovered from pooled draining LN (ILN+PLN) but none were present in inflamed tissue (Figure 4.13). In contrast, a marked number of BMMac were recovered from the inflamed tissues but no transferred cells were detected in draining LN (Figure 4.12 A). Moreover, *S. typhimurium* infection enhanced this effect by increasing the number of cells in inflamed joints. An absence of either BMDC or BMMac in bone marrow excluded the possibility that the cells were derived from the bone marrow of digested inflamed tissues. Although it is possible that transferred cells reach the inflamed joints via the blood stream, we did not find any transferred cells in blood samples in one experient (data not shown). This may be due to the time point (2 days after transfer) at which we acquired blood samples being too late to capture the transferred cells in the blood stream; additionally, the small blood volume analysed could limit our ability to detect transferred cells. Therefore, it would be of interest to further investigate the route by which transferred cells migrate to inflamed tissues.

Despite the low number of BMMac recovered in inflamed tissues, they appeared to be a real population (Figure 4.12 B). The small number of recovered BMMac could be related to the technical difficulty in obtaining cells from limbs. Cells were isolated from inflamed paws by enzyme digestion alone. Further purification by methods such as Percoll treatment could improve the recovery cells from those inflamed tissues.

The striking ability of BMMac, but not BMDC to migrate to inflammatory areas indicates that macrophages may play a more important antigen transportation role than DC in the pathogenesis of ReA. ReA is characterised by chronic inflammation of joints. This is due to persistence of nonviable bacteria and their products (such as LPS), which leads to the recruitment of leukocytes, including mononuclear phagocytes, into the inflamed synovium (Colmegna et al., 2004). We have demonstrated in our mouse model that recruitment of macrophages to the inflammatory joints occurs. Some studies have investigated the mechanisms behind this pattern of migration of macrophages. It has been shown that a significant number of adoptively transferred F4/80+ Mac-1+ splenocytes are able to migrate into the synovium of collagen-induced arthritis (Nanki et al., 2004). Furthermore, in that study, it was demonstrated that treatment with anti-fractalkine significantly reduced the migration of the Mac-1+ splenocytes into the synovium, resulting in the amelioration of arthritis. By testing expression of CX3CR1 on transferred macrophages and fractalkine (unique ligand of CX3CR1) expression in the synovium of collagen-induced arthritis mice, they found that the interaction of FKN-CX3CR1 partially mediated this specific migration of macrophages. Results from atherothesclerosis studies

may also reveal potential mechanisms involved in regulating macrophage migration. In this regard, two pairs of chemokines and their receptors MCP-1 (CCL2) (Gu et al., 1998) (Gosling et al., 1999)-CCR2 (Boring et al., 1998; Dawson et al., 1999) and IL8 (CXCL8)-CXCR2 (Boisvert et al., 1998), were shown to be important. Accordingly, an increased expression of IL-8 and MCP-1α is observed in inflamed knees of rabbits with induced acute experimental arthritis (Lopez-Armada et al., 2002). Earlier studies have shown that fluorescently labelled resident peritoneal macrophages, but not thioglycollate-elicited macrophages are able to migrate to the inflamed peritoneal cavity following intravenous adoptive transfer (Rosen and Gordon, 1990). This is mediated through a complement receptor 3 (CR3)-dependent mechanism, as this effect could be abolished by *in vitro* preincubation of labelled cells or *in vivo* treatment of the recipient with 5C6, an anti-mouse CR3 monoclonal antibody. However, in the absence of an inflammatory stimulus there was no migration of cells to the peritoneal cavity, and fluorescently labelled macrophages accumulated in the red pulp and marginal zone of the spleen. Moreover, migration of macrophages required viable injected cells as cells fixed with paraformaldehyde did not migrate and were cleared in the pulmonary microvasculature (Rosen and Gordon, 1990). In this context, it would be useful to examine whether in our hands BMMac could still migrate into the peripheral tissues in the absence of inflammation. Analysis of the migration of fixed cells would also reveal whether viable cells were required.

4.4 Conclusion

Using adoptive transfer of fluorescently labelled cells by i.p. injection, we showed that under normal conditions, *S. typhimurium* infected BMDC migrate to the spleen and MLN and emigrate out of the peritoneal cavity. Conversely, *S. typhimurium* infected BMMac were not retrieved in increased numbers compared to uninfected cells from any organs apart from PPs, but their number was significantly reduced in the peritoneal cavity. Intracellular bacteria were carried to the spleen and liver by *S. typhimurium* infected BMDC and BMMac, suggesting their potential role in bacterial transportation during *S. typhimurium* infection. However, in a *Salmonella* induced inflammatory arthritis model, BMDC were found in draining LNs rather than inflamed limbs, whereas BMMac, especially after *S. typhimurium* infection, were found in inflamed limbs, but not in draining LNs.

Chapter 5: Investigation of mechanisms behind distinct migration profile of C5 (TS) infected BMDC and BMMac

5.1 Introduction

Immature DC are located in most tissues and play a sentinel role in peripheral organs, where they continuously sample their environment. Upon triggering by microbial infection or their products, by inflammatory cytokines or by tissue damage, they move to secondary lymphoid organs where they meet T cells (de Saint-Vis et al., 1998). The fine positioning within the tissues of the different DC subsets and their migration upon encountering stimulations are tightly regulated by a variety of molecules directing cell trafficking, among which chemokines have been shown to play a predominant role (Caux et al., 2000). Chemokines exert their function through interactions with their corresponding receptors, which are a family of G protein-coupled receptors. By modulation of their expression on the surface of cells, chemokine receptors are considered as crucial factors for directing the movement of cells (D'Ambrosio et al., 2003).

Two chemokine receptors (CCR7 and CCR6) were chosen to be studied here. Upregulation of CCR7 upon DC maturation has been demonstrated as the main factor governing skin DC migration to SLO under inflammatory and steady-state conditions (Ohl et al., 2004). CCR6 is one of the inflammatory chemokine receptors expressed on immature DC (Schutyser et al., 2003), and is the

receptor of chemokine CCL20/MIP-3α. In DC, CCR6 expression is restricted to skin and gut epithelium (Sierro et al., 2001).

As ubiquitous cells residing in the majority of tissues and accumulated in areas of inflammation, macrophage trafficking is also regulated by chemokines secreted by tissue cells or infiltrated leukocytes through the interaction with an array of chemokine receptors (Baggiolini and Loetscher, 2000; Rossi and Zlotnik, 2000). For example, modulation of CCR5 expression in macrophages is important for their activation and recruitment at inflammatory sites (Bosco et al., 2004).

Previous studies have shown that maturation after exposure to *S. typhimurium* confers on BMDC the ability to be recruited towards the CCR7 ligands CCL19 and CCL21, but not to the CCR6 ligand CCL20 *in vitro* (Cheminay et al., 2002). Moreover, a correlation between switch in chemokine receptor expression and the *in vivo* migration pattern of DC and macrophages has been suggested, but not been verified in a previous study (Jeannin et al., 2003). We have observed the differential migration pattern of DC and macrophages *in vivo* after exposure to *S. typhimurium in vitro* in chapter 4. In this chapter, we set out to explore the possible mechanisms behind these distinct migration patterns, focusing mainly on the role of chemokine receptors CCR7 and CCR6. Furthermore, the role of chemokines in the migration of *S. typhimurium* infected DC *in vivo* was investigated.

5.2 Results

5.2.1 Detection of apoptosis of S. typhimurium infected BMMac and BMDC

Since unlike BMDC, no increased number of C5 (TS) infected BMMac were recovered in organs examined apart from PPs, together with the greatly reduced number of them found in PL compared to uninfected cells, it was suspected that this effect maybe due to the cell death *Salmonella* could have potentially induced (Chen et al., 1996a; Monack et al., 1996). To examine whether BMDC and BMMac exposed to the *S. typhimurium* strain C5 (TS) underwent apoptotic cell death, and to determine whether this could partially explain their differential *in vivo* migration, apoptosis was detected using annexin V staining of C5 (TS) infected BMDC and BMMac. Firstly, the level of apoptosis on infected cells at various time points after C5 (TS) infection was tested. Figure 5.1 A shows that less than 20% of apoptotic cells was observed among BMMac at 24, 48 and 72 hours post-infection. C5 (TS) infection did not induce more macrophages to undergo apoptosis, as uninfected and C5 (TS) infected cells showed a comparable level of apoptosis. A similar level of apoptosis (<20%) was also observed for uninfected or C5 (TS) infected BMDC at 24 hours post-infection. At later time points (48 and 72 hours), however, differences between infected and uninfected BMDC were apparent. Although less than 20% of uninfected BMDC displayed signs of apoptosis, nearly 40% of C5 (TS) infected BMDC were apoptotic at 48 and 72 hours post-infection (right panel of Figure 5.1A).

Secondly, since CFSE labelling was utilised prior to adoptive transfer, the effect of CFSE labelling on cell death was considered as well. At 24 hours post-infection, infected cells were harvested and labelled with CFSE, and the level of apoptosis was then tested. Figure 5.1B shows that the level of apoptosis on either uninfected or C5 (TS) infected cells remained less than 20% for both BMMac and BMDC. Hence, compared to the cells without CFSE treatment, CFSE labelling did not cause a higher level of apoptosis at this time point for either cell type. To further test the effects of CFSE, CFSE labelled cells were seeded back to flasks and harvested at 72 hours post infection. No increase in apoptosis among BMMac was observed at this time point. In contrast, 60% of C5 (TS) infected BMDC compared to less than 20% of uninfected BMDC underwent apoptosis. In summary, BMDC, but not BMMac were induced to undergo apoptosis upon exposure to C5 (TS). CFSE labelling did not cause further apoptosis among BMMac, but this procedure did increase the level of apoptosis of BMDC at 72 hours post-infection.

5.2.2 Analysis of CCR7 and CCR6 expression on BMMac and BMDC upon S. typhimurium infection by RT-PCR

To determine whether infection with *S. typhimurium* would alter the expression of CCR7 and CCR6 on BMDC and BMMac, CCR7 and CCR6 gene expression was analysed at the mRNA level by RT-PCR. CCR7 and CCR6 expression was detected on uninfected BMDC. Upon *S. typhimurium* (C5 (TS) or SL3261) infection or LPS treatment, CCR7 expression was maintained, whereas, CCR6 expression was downregulated (Figure 5.2 upper panel). In contrast, CCR7

expression was not detected on uninfected BMMac. However, upon LPS stimulation or C5 (TS) infection, but not SL3261 infection, the expression of CCR7 on BMMac was detectable, especially on LPS treated BMMac. CCR6 showed a very faint band on uninfected BMMac samples, and expression became apparent upon LPS stimulation or *S. typhimurium* infection (Figure 5.2 bottom panel). Thus, RT-PCR data revealed that CCR7 expression was increased on BMMac upon *S. typhimurium* infection or LPS treatment, although differences were not detected for BMDC. CCR6 expression was downregulated on BMDC but slightly increased on BMMac after infection or LPS stimulation.

5.2.3 Quantification of CCR7 and CCR6 expression on BMDC and BMMac by real-time RT-PCR

Detection of chemokine receptor expression by RT-PCR is only qualitative. In order to accurately assess alterations in chemokine receptor expression on cells in response to *S. typhimurium*, a semi-quantitative method to analyse mRNA levels, namely, real-time RT-PCR was used. Figure 5.3 shows that in comparison to uninfected cells, CCR7 expression increased by 10 fold on BMDC upon C5 (TS) infection. The positive control, LPS treated BMDC also showed a large (16-fold) increase of CCR7 expression (Figure 5.3A). On the contrary, CCR6 expression was clearly downregulated on C5 (TS) infected (21 fold) and LPS treated BMDC (30 fold) compared to uninfected BMDC (Figure 5.3B). BMMac increased CCR7 expression by 6 fold and 8 fold (Figure 5.4A), and decreased CCR6 expression by 4 fold and 3 fold (Figure 5.4B) in response to C5 (TS) infection or LPS treatment respectively. Hence, the expression of

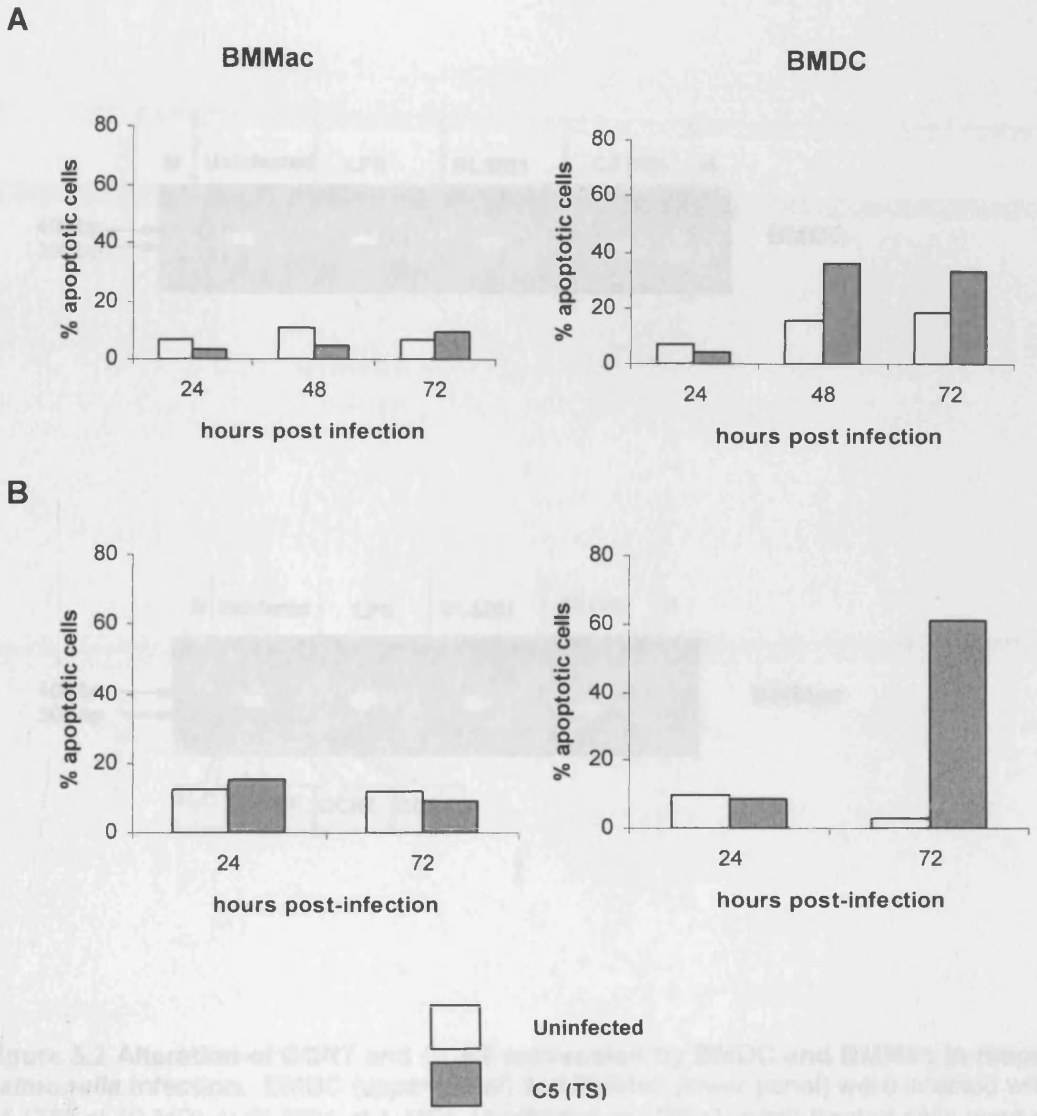


Figure 5.1 Levels of apoptosis of BMMac and BMDC after infection with C5 (TS). BMMac and BMDC were infected with C5 (TS) at 10 MOI. (A) Cells were harvested at time points indicated and stained with annexin V (Cy-chrome) and propidium iodide (PI). (B) At 24 hours post infection, all cells were harvested and labelled with CFSE. Half of them were directly stained with annexin V (Cy-chrome) and PI. The other half were seeded back to flask and further incubated for 48 hours (72 hours post-infection). These cells were then harvested and stained with annexin V (Cy-chrome) and PI. The percentage of apoptotic cells, which were annexin V (+) but PI (-), was determined by density plot analysis. Results are representative of three independent experiments.

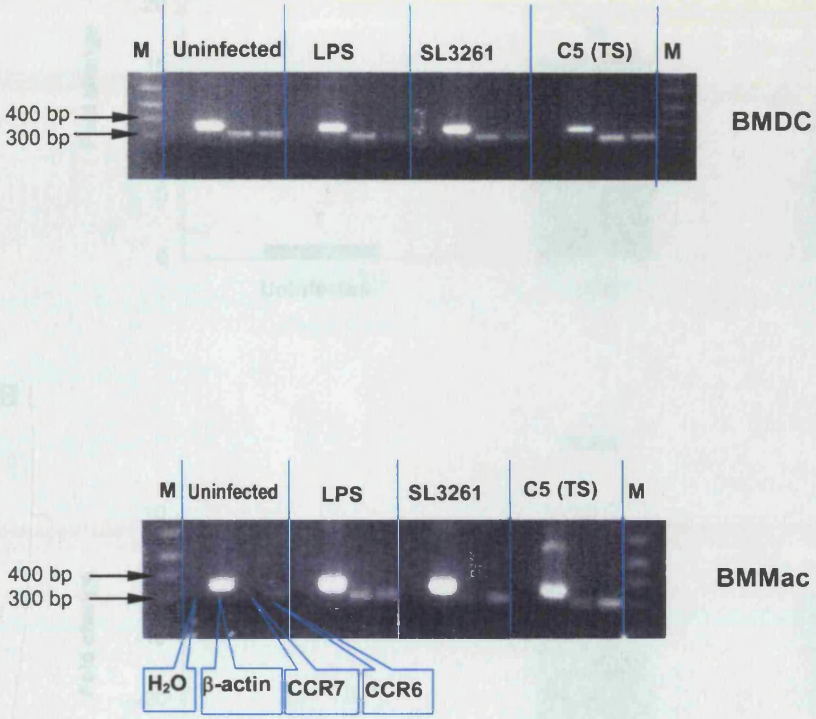


Figure 5.2 Alteration of CCR7 and CCR6 expression by BMDC and BMMac in response to *Salmonella* infection. BMDC (upper panel) and BMMac (lower panel) were infected with either C5 (TS) at 10 MOI or SL3261 at 1 MOI. Uninfected or LPS (1μg/ml) treated cells were used as controls. Twenty-four hours post-infection, RNA was extracted from harvested cells. CCR7 and CCR6 gene expression was analysed by RT-PCR. PCR products for each condition were loaded onto the gel as indicated. β-actin was conducted as a loading control. M, marker.

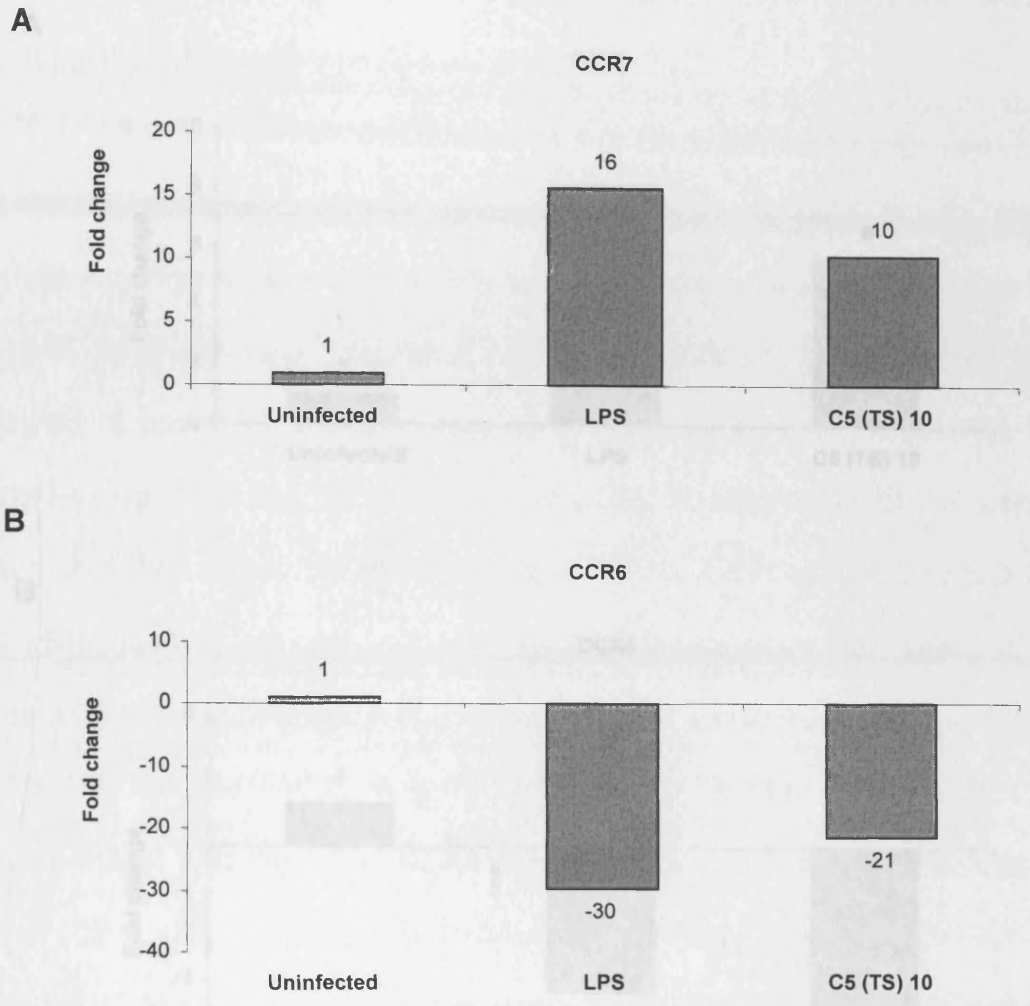


Figure 5.3 Semi-quantitative analysis of CCR7 and CCR6 expression in response to *Salmonella* infection in BMDC by real-time RT-PCR. BMDC were infected with C5 (TS) at 10 MOI. Uninfected and LPS treated cells were used as controls. Twenty-four hours post infection, cells were harvested and RNA was isolated from each sample. Expression of CCR7 (panel A) or CCR6 (panel B) was analysed by real-time RT-PCR. The fold change of expression was calculated according to "calibrator" (uninfected cells) and compared among samples. The value on the top of each bar is the actual fold change of each sample. Results are representative of 3 experiments.

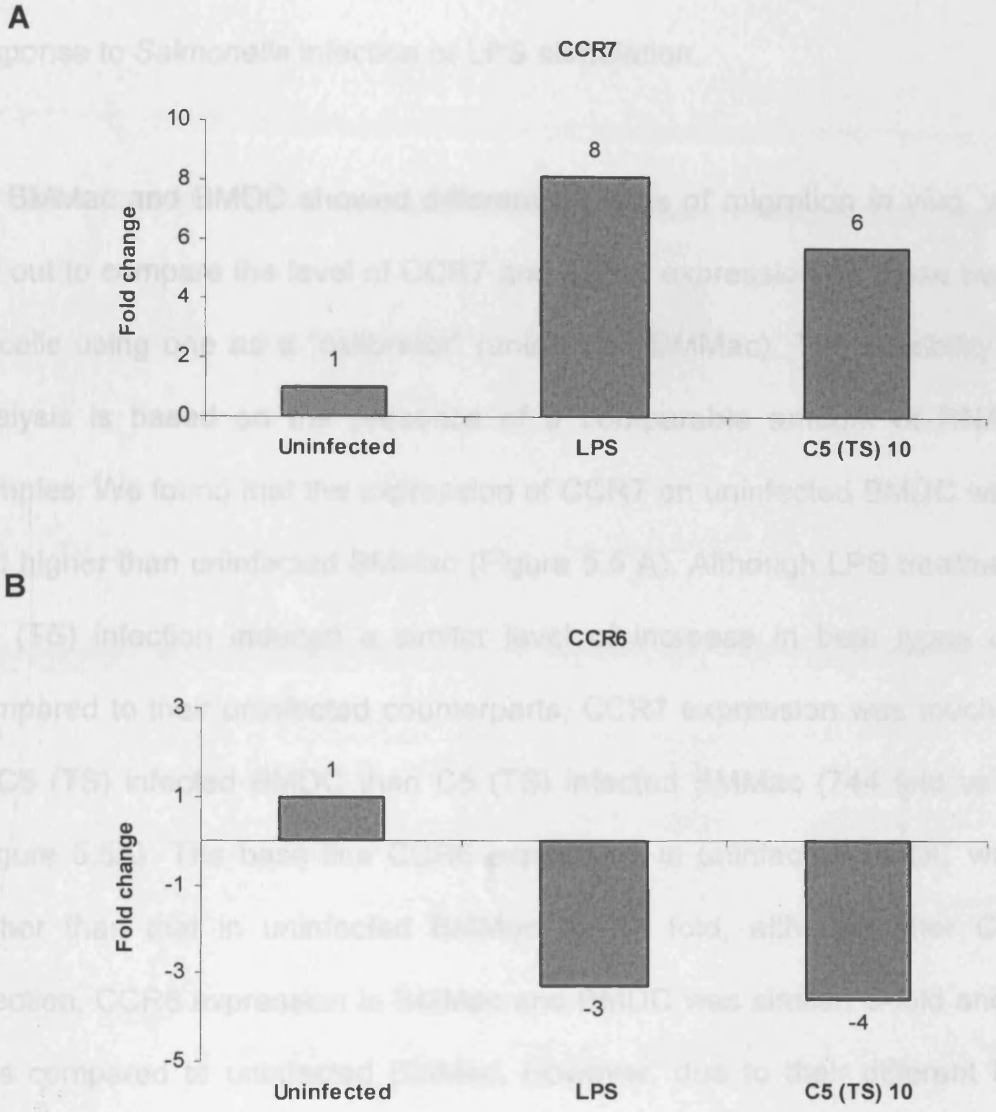


Figure 5.4 Semi-quantitative analysis of CCR7 and CCR6 expression in response to *Salmonella* infection in BMMac by real-time RT-PCR. BMMac were infected with C5 (TS) at 10 MOI. Uninfected and LPS treated cells were used as controls. Twenty-four hours post infection, cells were harvested and RNA was isolated from each sample. Expression of CCR7 (panel A) or CCR6 (panel B) was analysed by real-time RT-PCR. The fold change of expression of each sample was calculated according to "calibrator" (uninfected cells) and compared among samples. The value on the top of each bar is the actual fold change of each sample. Results are representative of 3 experiments.

CCR7 and CCR6 on BMDC and BMMac appeared to follow a similar pattern in response to *Salmonella* infection or LPS stimulation.

As BMMac and BMDC showed different patterns of migration *in vivo*, we next set out to compare the level of CCR7 and CCR6 expression on these two types of cells using one as a "calibrator" (uninfected BMMac). The feasibility of this analysis is based on the presence of a comparable amount of RNA in all samples. We found that the expression of CCR7 on uninfected BMDC was 241-fold higher than uninfected BMMac (Figure 5.5 A). Although LPS treatment and C5 (TS) infection induced a similar level of increase in both types of cells compared to their uninfected counterparts, CCR7 expression was much higher in C5 (TS) infected BMDC than C5 (TS) infected BMMac (744 fold vs 6 fold) (Figure 5.5A). The base line CCR6 expression in uninfected BMDC was also higher than that in uninfected BMMac by 13 fold, although after C5 (TS) infection, CCR6 expression in BMMac and BMDC was similar, 3-fold and 4-fold less compared to uninfected BMMac. However, due to their different level of base line expression, the extent of alteration induced by C5 (TS) infection was greater for BMDC than for BMMac (16-fold vs 5-fold) (Figure 5.5B). Taken together, upregulation of CCR7 and downregulation of CCR6 were observed on both types of cells in response to C5 (TS) infection, but due to the different base lines of expression in uninfected cells, CCR7 expression in C5 (TS) infected BMDC was much greater than BMMac, whereas, CCR6 expression after C5 (TS) infection was comparable.

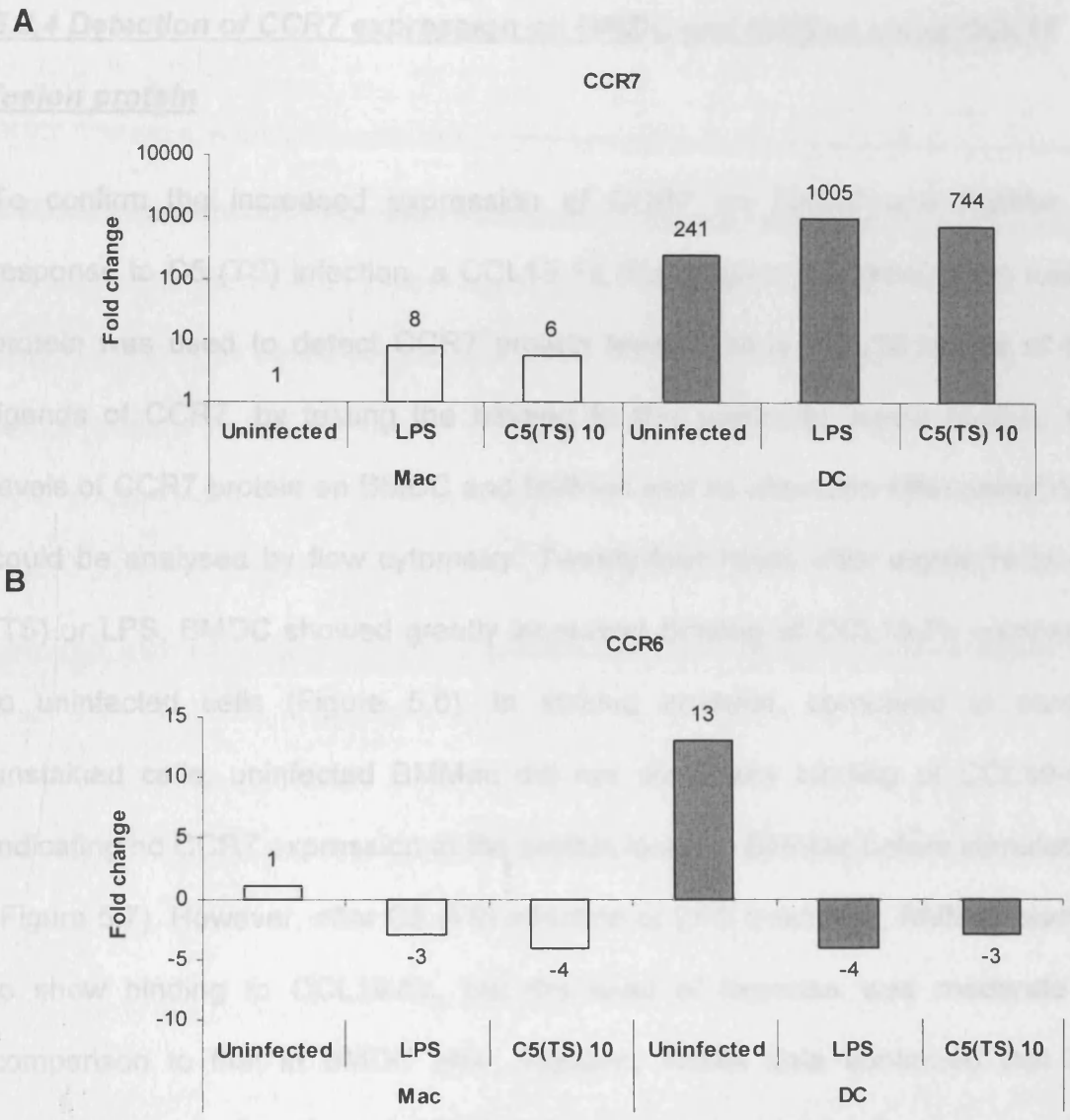


Figure 5.5 Comparison of CCR7 and CCR6 expression between BMDC and BMMac. BMMac (white bars) and BMDC (grey bars) were infected with C5 (TS) at 10 MOI. Uninfected and LPS treated cells were used as controls. Cells were harvested 24 hours post-infection and RNA was isolated from each sample. Expression of CCR7 (panel A) or CCR6 (panel B) was analysed by real-time RT-PCR. The fold change of expression was calculated according to "calibrator" (uninfected BMMac) and compared among samples. The value on the top of each bar is the actual fold change of each sample. Results are representative of 3 experiments.

5.2.4 Detection of CCR7 expression on BMDC and BMMac using CCL19 fusion protein

To confirm the increased expression of CCR7 on BMDC and BMMac in response to C5 (TS) infection, a CCL19-Fc (Fc, fragment crystallizable) fusion protein was used to detect CCR7 protein levels. Since CCL19 is one of the ligands of CCR7, by testing the binding to this particular fusion protein, the levels of CCR7 protein on BMDC and BMMac and its alteration after stimulation could be analysed by flow cytometry. Twenty-four hours after exposure to C5 (TS) or LPS, BMDC showed greatly increased binding of CCL19-Fc compared to uninfected cells (Figure 5.6). In striking contrast, compared to control unstained cells, uninfected BMMac did not show any binding of CCL19-Fc, indicating no CCR7 expression at the protein level on BMMac before stimulation (Figure 5.7). However, after C5 (TS) infection or LPS treatment, BMMac started to show binding to CCL19-Fc, but the level of increase was moderate in comparison to that in BMDC after infection. These data confirmed that the expression and alteration of CCR7 on these two types of cells at the protein level were consistent with its change at the mRNA level.

5.2.5 Functional test of chemokine receptors on BMDC and BMMac by chemotaxis assay

Following the detection of CCR7 up-regulation with concomitant CCR6 downregulation on BMDC upon C5 (TS) infection, the changes in migratory capacity of BMDC towards the chemokines CCL19 and CCL20 after C5 (TS) infection were assessed by *in vitro* chemotaxis assay, with LPS treated cells as

a positive control. Figure 5.8 shows that uninfected BMDC exhibited a low level of migratory capacity (10%) towards CCL19 compared to that of medium alone (5%). This was consistent with the previous data that showed CCR7 expression on uninfected BMDC. After exposure to C5 (TS) or LPS, the capacity of BMDC to migrate towards CCL19 was significantly enhanced, as more than 30% of cells migrated compared to less than 10% of cells to medium alone. Uninfected BMDC migrated towards CCL20, although the capacity was low. Approximately 10% of cells were able to migrate towards CCL20 compared to 5% background migration in medium alone. However, after exposure to C5 (TS) or LPS, BMDC lost their ability to migrate towards CCL20 (Figure 5.9). These results confirmed that both CCR7 up-regulation and CCR6 downregulation on BMDC were functional, which conferred a gain in responsiveness to the chemokine CCL19 and loss in responsiveness to the chemokine CCL20 after C5 (TS) infection or LPS treatment.

5.2.6 Preincubation with CCL19 induce desensitisation of CCR7 on BMDC

G protein coupled receptors become desensitised after stimulation with their ligands, resulting in a failure of cells to migrate towards their ligands upon subsequent exposure (Kohout et al., 2004). In order to test if the *in vivo* migration of C5 (TS) infected BMDC to SLO was related to CCR7 upregulation upon infection, we decided to evaluate the consequences of *in vitro* desensitisation of CCR7 with its ligand CCL19. Firstly, the conditions for CCR7 desensitisation were optimised using the chemotaxis assay. Figure 5.10 shows that after 45 minutes incubation with various concentrations of

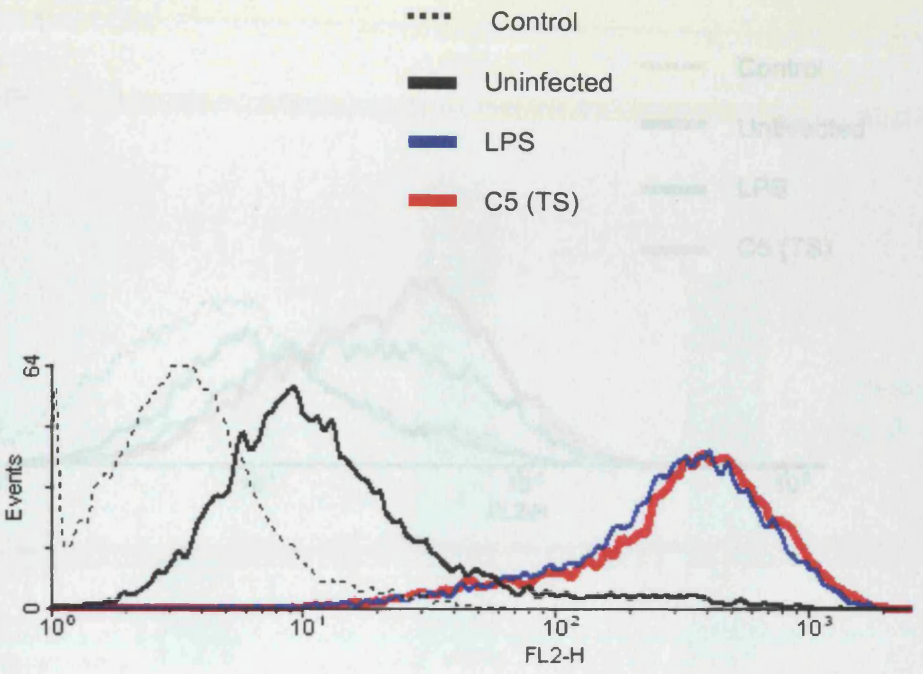


Figure 5.6 Expression of CCR7 protein and its alteration in response to C5 (TS) infection on BMDC detected by CCL19 fusion protein. BMDC were infected with C5 (TS) at 10 MOI. Uninfected and LPS treated DC were used as controls. Twenty-four hours post infection, cells were harvested and incubated with the CCL19-Fc recombinant protein, which was a fusion of mouse CCL19 and the human IgG1 constant region, followed by goat anti-human IgG (PE). For control staining, cells were incubated in the absence of fusion protein. Histograms show staining of DC treated as indicated.

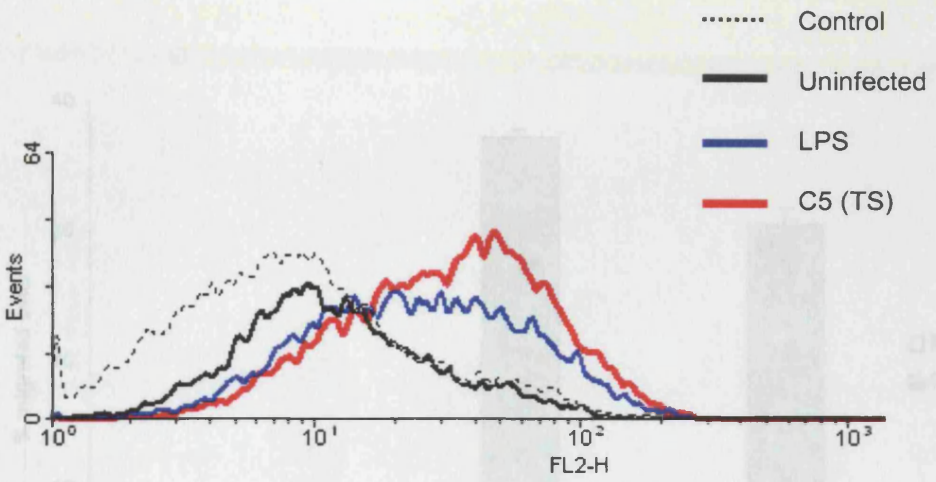


Figure 5.7 Expression of CCR7 protein and its alteration in response to C5 (TS) infection on BMMac detected by CCL19 fusion protein. BMMac were infected with C5 (TS) at 10 MOI. Uninfected and LPS treated cells were used as controls. Twenty-four hours post infection, cells were harvested and incubated with the CCL19-Fc recombinant protein, which was a fusion of mouse CCL19 and the human IgG1 constant region, followed by goat anti-human IgG (PE). For control staining, cells were incubated in the absence of fusion protein. Histograms show staining of BMMac treated as indicated.

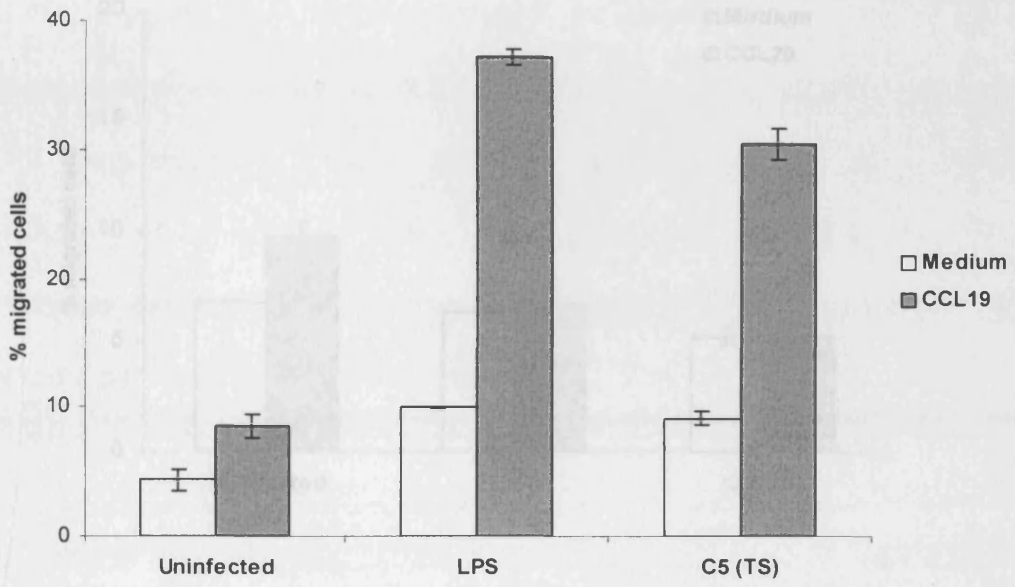


Figure 5.8 Migration of BMDC towards CCL19 (MIP-3β) in response to C5 (TS) infection. BMDC were infected with C5 (TS) at 10 MOI. Uninfected and LPS treated cells used as controls for infection. The functional migration of DC towards CCL19 (30 nM) 24 hours post infection was analysed by a chemotaxis assay, with medium alone as a control for specific migration. Migration was measured by the percentage of migrated cells. Data represent mean values of duplicates performed in transwells. Results are representative of two experiments.

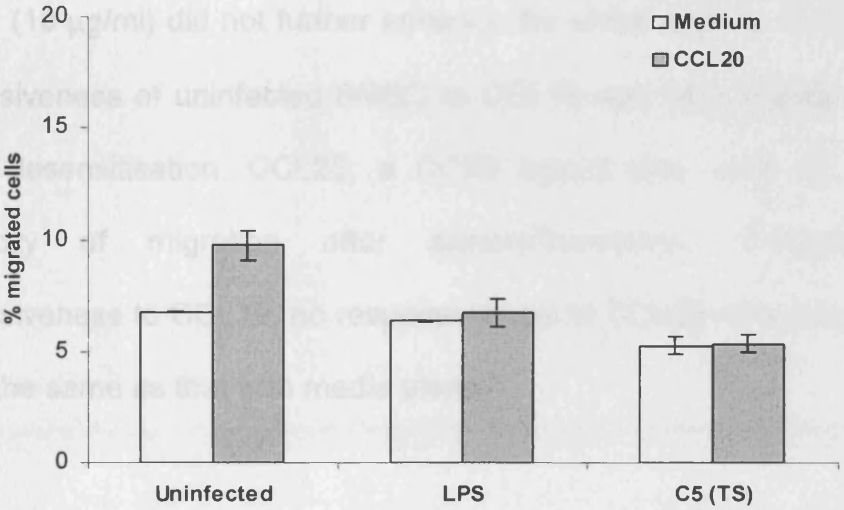


Figure 5.9 Migration of BMDC towards CCL20 (MIP-3α) in response to C5 (TS) infection. BMDC were infected with C5 (TS) at 10 MOI. Uninfected and LPS treated cells used as controls for infection. The functional migration of DC towards CCL20 (100 ng/ml) 24 hours post infection was analysed by a chemotaxis assay, with medium alone as a control for specific migration. Migration was measured by the percentage of migrated cells. Data represent mean values of duplicates performed in transwells. Results are the representative of two experiments.

CCL19, the migratory capacity of C5 (TS) infected BMDC towards CCL19 was greatly diminished. Optimal desensitisation was obtained by incubation of the cells with CCL19 at a concentration of 5 μg/ml, and a higher concentration of CCL19 (10 μg/ml) did not further enhance the effect (Figure 5.10). Notably, the responsiveness of uninfected BMDC to CCL19 was also slightly reduced after CCR7 desensitisation. CCL20, a CCR6 ligand was used as a control for specificity of migration after desensitisentation. Compared to the responsiveness to CCL19, no responsiveness to CCL20 was observed with the levels the same as that with media alone.

5.2.7 Decreased migration of BMDC to spleen and MLN caused by CCL19-mediated CCR7 desensitisation

In order to test if the *in vivo* migration of C5 (TS) infected BMDC to SLO was dependent on CCR7, C5 (TS) infected BMDC were desensitised by incubation with 5 μg/ml CCL19 prior to adoptive transfer (cells incubated with PBS served as the non-desensitised control). In parallel, a small fraction of transferred cells was left to set up a chemotaxis assay to ascertain that the transferred cells were indeed desensitised. Figure 5.11 shows that compared to more than 30% of non-desensitised cells migrating towards CCL19 in a chemotaxis assay, the ability of desensitised cells to migrate to CCL19 was greatly reduced, to just over 10% (Figure 5.11). These results indicated that the desensitised cells used in *in vivo* transfer lost much of their responsiveness to CCL19.

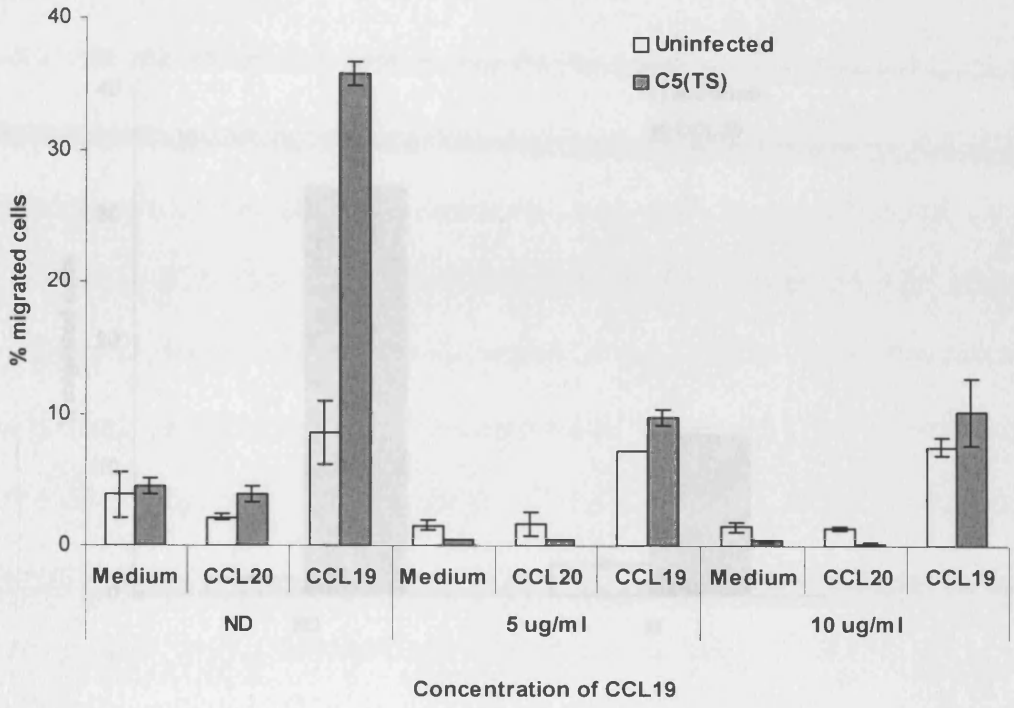


Figure 5.10 Selection of the concentration of CCL19 required for DC *in vitro* desensitisation. BMDC were infected with C5 (TS) at 10 MOI with uninfected cells used as a control. Twenty-four hours after infection, DC were harvested and split into 3 parts, which were incubated at 37°C for 1 hour in the presence of PBS, 5 μg/ml or 10 μg/ml of CCL19 respectively. After washing off the residual chemokine, cells were seeded into the upper compartment of transwells with CCL19 (30 nM), CCL20 (100 ng/ml) or medium alone in the bottom compartment. Migration was measured by the percentage of migrated cells. Data represent mean values of duplicates. Results are representative of two experiments. ND, non-desensitised.

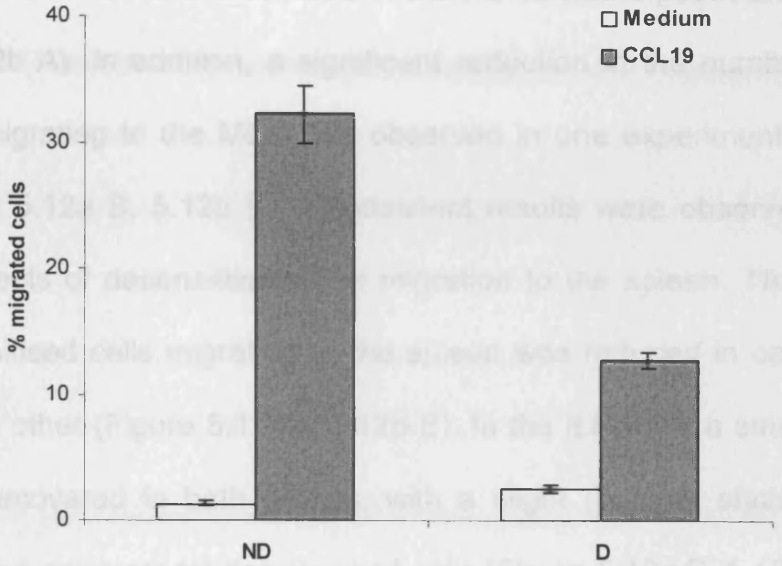


Figure 5.11 *In vitro* assessment of the desensitisation of DC used for adoptive transfer. BMDC were infected with C5 (TS) at 10 MOI. Twenty-four hours after infection, cells were harvested. Half were incubated with 5 μg/ml CCL19 (indicated as desensitised, D), while the other half were incubated with PBS only (indicated as non-desensitised, ND). Following incubation at 37°C for 1 hour, cells were labelled with CFSE before washing 3 times with PBS. Some cells were set up in the transwell assay before the majority of them were adoptively transferred to mice. Data are expressed as the mean percentage of migrated cells for duplicate wells.

The migration pattern of desensitised and non-desensitised C5 (TS) infected BMDC was compared in two independent experiments. In both, a comparable number of cells were recovered in the PL 48 hours post-transfer (Figure 5.12a A, 5.12b A). In addition, a significant reduction in the number of desensitised cells migrating to the MLN was observed in one experiment but not the other (Figure 5.12a B, 5.12b B). Inconsistent results were observed with respect to the effects of desensitisation on migration to the spleen. Thus, the number of desensitised cells migrating to the spleen was reduced in one experiment, but not the other (Figure 5.12aB, 5.12b B). In the ILN, only a small number of cells were recovered in both groups, with a slight (but not statistically significant) reduction apparent for desensitised cells (Figure 5.12a B, 5.12b B).

Taken together, the results from the two desensitisation experiments indicated that the migration of C5 (TS) infected BMDC toward MLN was inhibited significantly by *in vitro* CCR7 desensitisation; a firm conclusion on whether migration of the same cells to the spleen was also inhibited by CCR7 desensitisation could not be made at this stage.

5.2.8 Differential dependence on CCR7 in mediating DC migration to spleen and MLN

Since results generated using *in vitro* desensitisation were inconsistent, we decided to use a potentially more definitive model to assess the role of CCR7, namely CCR7 deficient mice (denoted as CCR7 knockout (KO)) described earlier ((Forster et al., 1999). Bone marrow cells isolated from CCR7 KO mice

(BALB/c background) were kindly provided by Dr. Martin Lipp (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany). DC were generated from bone marrow cells isolated from CCR7 KO or BALB/c mice. Infection and adoptive transfer procedures were then performed as in previous experiments, and the number of CFSE labelled cells in PL, spleen, MLN and ILN were compared between two groups 48-hours post-transfer.

Two independent experiments were performed with similar results. A small but significant reduction was observed in the number of CFSE labelled cells recovered from the PL of mice receiving CCR7 KO DC compared to BALB/c DC (Figure 5.13A). In contrast, similar numbers of CCR7 KO and WT DC were recovered from the spleens of recipient mice, indicating that CCR7 was not required for migration to this organ. Strikingly, however, virtually no CCR7 KO DC migrated to MLN or ILN (Figure 5.13B); for MLN, the difference between WT and CCR7 KO DC was highly statistically significant ($p<0.001$). Therefore, these data indicate a strict requirement for CCR7 in guiding migration of *S. typhimurium* infected BMDC to LN, but not spleen.

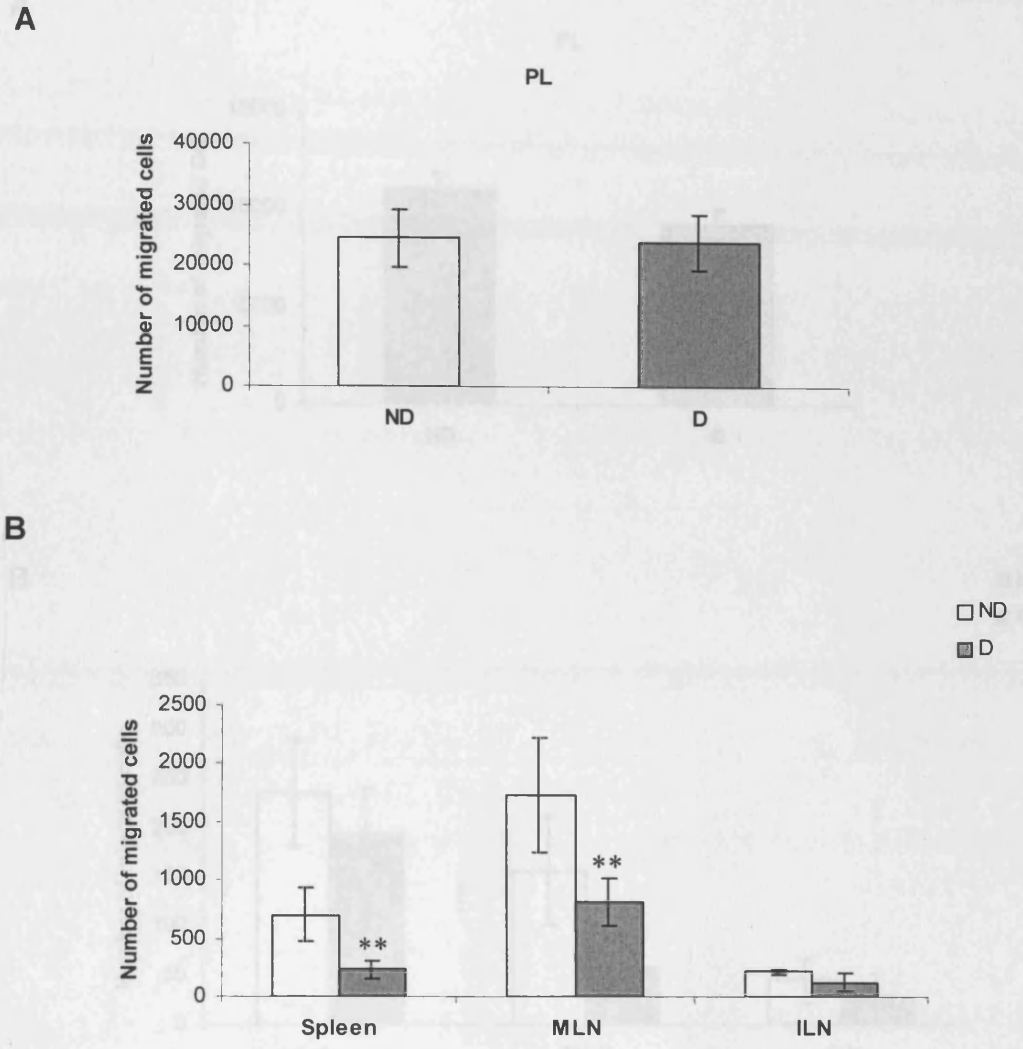


Figure 5.12a Effect of *in vitro* CCR7 desensitisation on DC migration to lymphoid organs (Experiment 1). BMDC were infected with C5 (TS) at 10 MOI. Twenty-four hours after infection, cells were harvested. Half were incubated with 5 μg/ml CCL19 (indicated as desensitised, D), while the other half were incubated with PBS only (indicated as non-desensitised, ND). Following incubation at 37°C for 1 hour, cells were labelled with CFSE before washing 3 times with PBS. Cells were then adoptively transferred to HLA-B27/β_2m mice by intraperitoneal injection (3 mice for ND group and 4 for D group). Forty-eight hours post-transfer, CFSE labelled cells were enumerated in suspensions from peritoneal lavage (PL), spleen, mesenteric lymph nodes (MLN) and inguinal lymph nodes (ILN) by flow cytometry. (A) Number of CFSE labelled cells in PL. (B) Number of CFSE labelled cells in spleen, MLN and ILN. ** $p<0.05$ vs ND (Two-sample student's T-test).

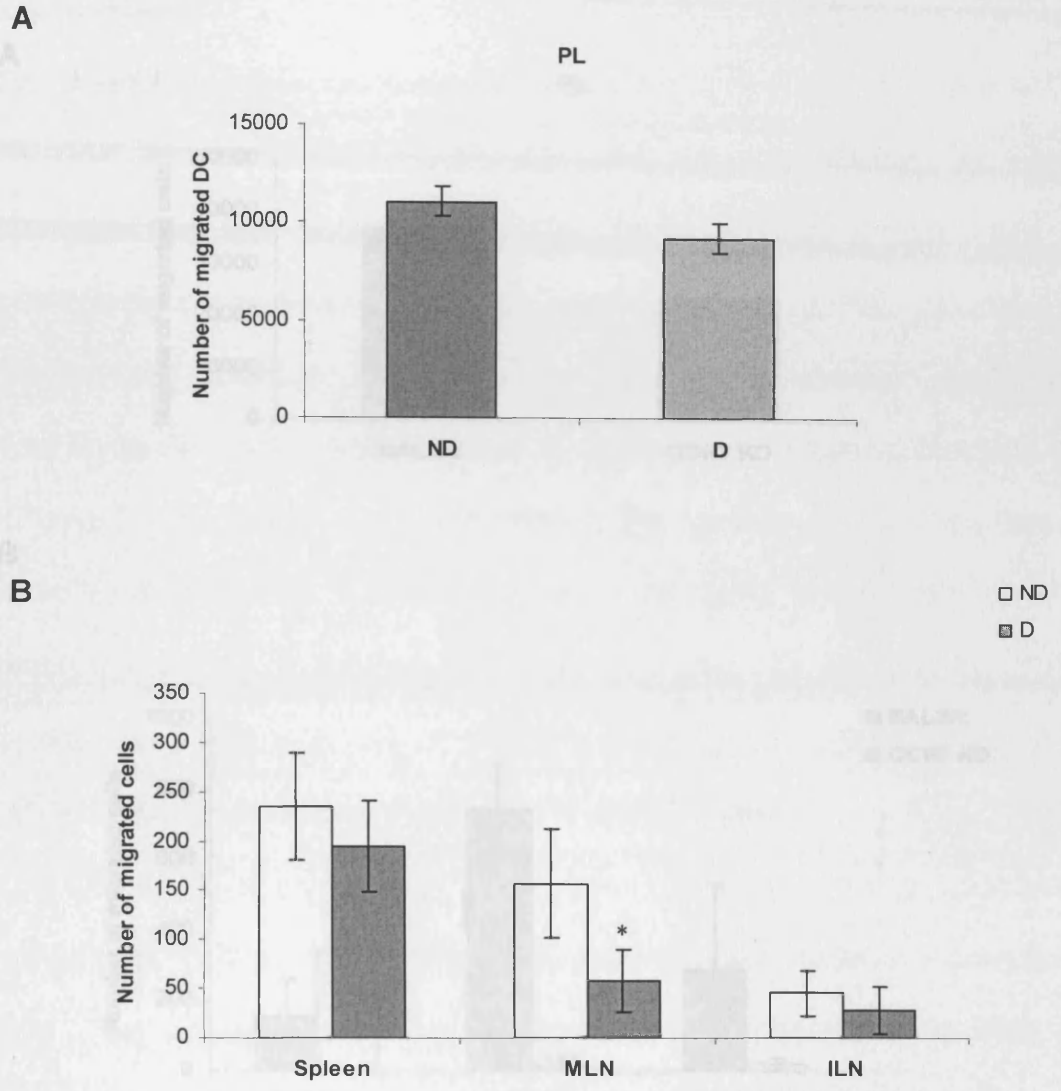


Figure 5.12b Effect of *in vitro* CCR7 desensitisation on DC migration to lymphoid organs (Experiment 2). BMDC were infected with C5 (TS) at 10 MOI. Twenty-four hours after infection, cells were harvested. Half were incubated with 5 μg/ml CCL19 (indicated as desensitised, D), while the other half were incubated with PBS only (indicated as non-desensitised, ND). Following incubation at 37°C for 1 hour, cells were labelled with CFSE before washing 3 times with PBS. Cells were then adoptively transferred to HLA-B27/β_2m mice by intraperitoneal injection (3 mice of each group). Forty-eight hours post-transfer, CFSE labelled cells were enumerated in suspensions from peritoneal lavage (PL), spleen, MLN and inguinal LN (ILN) by flow cytometry. (A) Number of CFSE labelled cells in PL. (B) Number of CFSE labelled cells in spleen, MLN and ILN. * p=0.05 vs ND (Two-sample student's T-test).

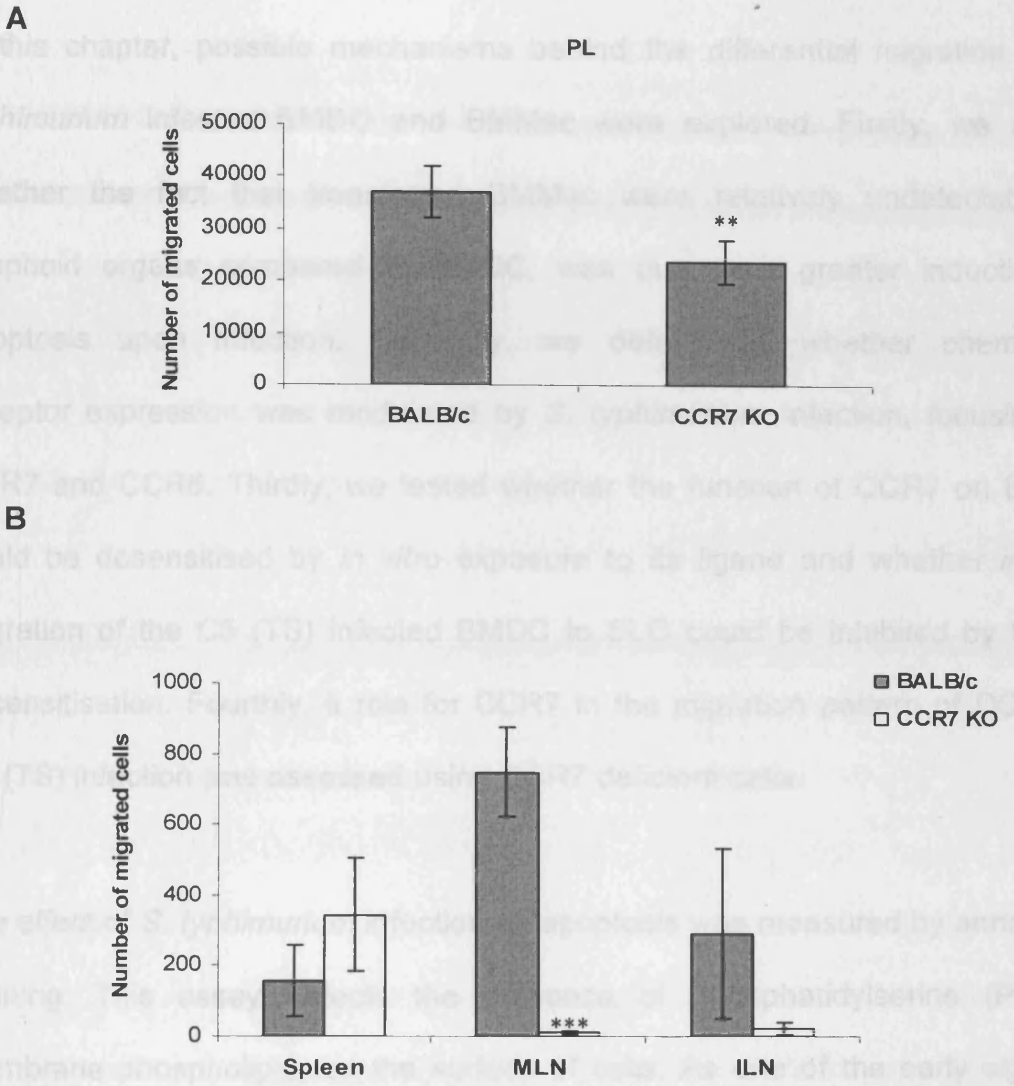


Figure 5.13 Differential dependence of CCR7 in mediating DC migration to spleen and lymph nodes. BMDC were propagated by 7-day culture of bone marrow cells isolated from BALB/c or CCR7 KO mice in the presence of GM-CSF. CCR7 KO bone marrow cells were generously provided by Dr. Martin Lipp from Germany. At day 8, adherent cells were infected with C5 (TS) at 10 MOI. Cells were harvested and labelled with CFSE 24 hours after infection. CFSE labelled cells (1×10^7/mouse) were then adoptively transferred to BALB/c recipient mice by intraperitoneal injection (4 mice of each group). Forty-eight hours post-transfer, CFSE labelled cells were enumerated in suspensions from PL, spleen, MLN and ILN by flow cytometry. (A) Number of CFSE labelled cells in PL. (B) Number of CFSE labelled cells in spleen, MLN and ILN. Results are representative of two independent experiments with the similar results. ** $p<0.01$, *** $p<0.001$ vs BALB/c (Two-sample student's T-test).

5.3 Discussion

In this chapter, possible mechanisms behind the differential migration of *S. typhimurium* infected BMDC and BMMac were explored. Firstly, we asked whether the fact that transferred BMMac were relatively undetectable in lymphoid organs compared to BMDC, was due to a greater induction of apoptosis upon infection. Secondly, we determined whether chemokine receptor expression was modulated by *S. typhimurium* infection, focusing on CCR7 and CCR6. Thirdly, we tested whether the function of CCR7 on BMDC could be desensitised by *in vitro* exposure to its ligand and whether *in vivo* migration of the C5 (TS) infected BMDC to SLO could be inhibited by CCR7 desensitisation. Fourthly, a role for CCR7 in the migration pattern of DC after C5 (TS) infection was assessed using CCR7 deficient cells.

The effect of *S. typhimurium* infection on apoptosis was measured by annexin V staining. This assay detects the presence of phosphatidylserine (PS), a membrane phospholipid, on the surface of cells. As one of the early signs of apoptosis, PS is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, thus serving as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis.

We demonstrated that BMMac (either uninfected or C5 (TS) infected) did not show a greater tendency to undergo apoptosis than BMDC (Figure 5.1). In fact, lower rate of cell death of macrophages compared to DC observed in our *in*

vitro system suggests that macrophages may not die in large numbers after adoptive transfer. Thus, these results raised the possibility that the poor detection of macrophages after transfer might be due to their ability to migrate to areas such as peripheral tissues, making them hard to be detected. Our results supported this possibility, since transferred macrophages but not DC indeed were able to migrate to the inflamed peripheral tissues in small numbers (Figure 4.12). Whether this migration also occurs under non-inflammatory conditions is worthy of further investigation.

The migration profile of macrophages observed here is consistent with other studies. Geissman et al (Geissmann et al., 2003) studied migration of macrophage precursors, a subpopulation of monocytes whose phenotype defined as Gr-1^{+}CX$_3$CR1^{low}CCR2^{+}CD62L^{+}. These cells were almost undetectable in all tissues analysed except the spleen one day after adoptive transfer into naive mice. However, if these cells were transferred into animals with experimentally induced peritonitis, they infiltrated the inflammatory area, ie, the peritoneal cavity. The relationship between our BMMac and the particular population of monocytes described in their study would an interesting issue to consider.

Various other studies have examined the migration of macrophages after adoptive transfer. In one, CFSE labelled macrophages stimulated with a microbial product *in vitro* could not be detected at any time point analysed after injection into the footpads of mice (Jeannin et al., 2003). Another paper reported that viable cells accumulate predominantly in the spleen and liver while non-

viable cells are rapidly trapped in the pulmonary microvasculature (Rosen and Gordon, 1990) after i.v transfer. It is possible that viable cells could also be trapped in the pulmonary microvasculature, but to a lesser extent than non-viable cells.

BMDC were induced to undergo apoptosis to a greater extent than macrophages when left in culture for two days post infection. Supporting our results, it has been previously shown that *S. typhimurium* can induce BMDC to undergo apoptosis in a dose-dependent manner (Yrlid et al., 2001b). A contributing factor may be cultured BMDC have reached their final stage of maturation by 7-8 days (Lutz et al., 1999). Indeed, in our study, we found that DC were sensitive to even very minor procedures such as changing the medium and pipetting, which would mature DC, demonstrated by 4-8 fold upregulation of CCR7 mRNA (data not shown). Furthermore, after being induced to mature by treatment with TNF-α, 40% of BMDC appear apoptotic following 6 hours of culture in medium alone. The implication is that mature DC are relatively short-lived, at least *in vitro* (Sanchez-Sanchez et al., 2004).

Results from cross-transfer experiments suggested that transferred BMDC also undergo some apoptosis *in vivo*. In the peritoneal lavage, nearly 15% of uninfected BMDC were engulfed by endogenous cells, indicated by their double staining for CFSE and HLA-ABC. *S. typhimurium* infection augmented this effect by another 5% (Figure 4.3). Signs of apoptosis on transferred cells would be sensed by endogenous cells such as peritoneal macrophages, resulting in the apoptotic cells being phagocytosed. Thus, about 15% of uninfected BMDC

in contrast to 20% of C5 (TS) infected BMDC might undergo apoptosis after adoptive transfer. Finally, CFSE labelling did not increase the level of the apoptosis on uninfected cells but enhanced the level of apoptosis on C5 (TS) infected cells suggesting a synergistic effect of CFSE labelling and C5 (TS) infection on BMDC.

Several lines of evidence suggest that DC reprogram their chemokine receptor expression profile during defined maturation stages (Fleming et al., 2003; Sato et al., 2001; Yanagawa and Onoe, 2003). Functional maturation is associated with loss of responsiveness to chemokines present at sites of inflammation and acquisition of a receptor repertoire that renders these cells responsive to signals that guide their localisation in lymphoid organs (Sozzani et al., 1999). As mentioned in Chapter 1, immature DC respond to a large spectrum of chemokines through specific receptors (Table 1.2).

Different immature DC subsets display unique sensitivity to certain chemokines, which allows them to be recruited to various peripheral tissues where they play a sentinel role. For instance, a previous study has reported that F4/80-B220-CD11c+ DC expressing CCR1 and CCR5 are mobilized rapidly into the circulation in mice injected with *propionibacterium acnes* to induce granulomas in the liver, and are recruited into inflammatory tissue by CCL3/MIP-1α, which binds to CCR1 and CCR5 (Yoneyama et al., 2001). CCL20/MIP-3α, produced by keratinocytes, is important in recruiting Langerhans cells (skin dendritic cells) to the epidermis through CCR6 (Charbonnier et al., 1999). Interestingly, it has been shown that *Salmonella*, but not indigenous bacteria of the intestinal flora

are able to upregulate expression of the CCL20 gene in human intestinal epithelial cell lines (Sierro et al., 2001). Such a mechanism could be instrumental in initiating adaptive immune responses in the gut upon *Salmonella* infection. Based on these observations, CCR6 was investigated in our study as a representative chemokine receptor expressed on immature DC.

After antigen uptake, inflammatory stimuli turn off the responsiveness of immature DC to chemokines specific for immature DC through receptor downregulation. Consequently, maturing DC escape from the local gradient of chemokines that immature DC respond to (Banchereau et al., 2000). Meanwhile, upon maturation, DC upregulate the chemokine receptor CCR7 (Yoshida et al., 1997), and acquire responsiveness to CCL19 and CCL21. The role of these two co-receptor chemokines in recruiting DC differs according to their site of expression. CCL21 is expressed on lymphatic vessels and directs maturing DC to leave inflamed tissues and enter the lymph stream (Gunn et al., 1998; Saeki et al., 1999). CCL19 and CCL21 are both secreted by the stromal cells in the T cell zones and are responsible for directing mature DC to enter SLO (Dieu et al., 1998; Ngo et al., 1998). Both chemokines are full agonists with similar receptor binding affinities and chemotactic potencies for CCR7 (Ott et al., 2004; Sullivan et al., 1999; Yoshida et al., 1998). The reason for the existence of two ligands for CCR7 is not clear.

Real-time RT-PCR results clearly showed that *S. typhimurium* infection drove BMDC and BMMac to alter expression of these two chemokine receptors, with CCR7 expression upregulated and CCR6 downregulated (Figure 5.3 and 5.4).

However, the presence of the bands on the gel from our RT-PCR results demonstrated that CCR7 and CCR6 were expressed on these two populations. This is inconsistent with a previous study showing CCR6 mRNA expression was not detectable by RT-PCR in immature and mature DC (Cheminay et al., 2002). Nevertheless, the detailed expression of CCR6 on different DC subsets was addressed using EGFP/CCR6 knock-in mice (EGFP expressed under the CCR6 promoter to visualise CCR6 expression) (Kucharzik et al., 2002). In this study, it was shown that most CD11b+ myeloid DC expressed CCR6, but that CD8α+ lymphoid DC are negative for CCR6; epidermal Langerhans cells expressed CCR6, but at lower levels than splenic myeloid DC. CD11c+ DC generated from culture of bone marrow precursors from the knock-in mice with GM-CSF also expressed CCR6. Therefore, our results agreed with the study of CCR6 knock-in mice, indicating that CCR6 is indeed expressed on BMDC. CCR6 expression on major DC subsets suggest that its ligand, CCL20, participates in determining the positioning of DC subsets, including DC recruited from bone marrow, in peripheral tissues.

Our real-time RT-PCR results confirmed that BMDC down-regulated CCR6 expression and coordinatedly upregulated CCR7 expression upon *S. typhimurium* infection or LPS treatment (Figure 5.4B). Due to the previous lack of an antibody against the murine CCR7 protein, CCR7 protein expression was measured using a CCL19-Fc fusion protein (Figure 5.6). These results indicated that increases in CCR7 mRNA were associated with increased surface expression of CCR7. Increased CCR7 protein expression on matured BMDC

has recently been shown using an anti-CCR7 antibody (Cheminay et al., 2002) (Ritter et al., 2004).

Our results showing a "switch" of chemokine receptors on DC are consistent with the studies of others (Moutaftsi et al., 2004). It has been suggested that the downregulation of CCR6 and upregulation of CCR7 underlies langerhans cell migration from skin to lymphoid tissue (Fleming et al., 2003). Similarly, CCR1 and CCR5 are downregulated upon DC maturation, whereas CCR7 is selectively upregulated facilitating their migration from peripheral tissues to regional lymph nodes (Yanagawa and Onoe, 2003). Studies from human monocyte derived DC further reveal the dynamic modulation of chemokine receptors on DC at different stages of development (Moutaftsi et al., 2004). These authors found that cell-surface expression of CCR1, CCR3 and CCR5 was increased during differentiation of immature DC from monocytes. In contrast, expression was decreased during the development of immature DC into mature DC, while CCR7 expression was detected on mature DC, but not on monocytes or immature DC (Sato et al., 2001). Moreover, DC also switched their functional migration towards different chemokines. That is, immature DC and their precursor monocytes, but not mature DC migrated in response to the inflammatory chemokine CCL5, whereas mature DC, but not monocytes or immature DC, migrated to CCL19 (Sato et al., 2001). In this respect, our study also demonstrated that the switch in chemokine receptors on BMDC was a functional switch. More mature, infected BMDC migrated towards CCL19, whereas downregulation of CCR6 expression resulted in a loss in ability to migrate towards the CCR6 ligand CCL20 (Figure 5.8-5.9). These results are

consistent with those of another study (Cheminay et al., 2002), in which BMDC exposured to *S. typhimurium* migrated not only towards CCL19 but also CCL21, another CCR7 ligand, but not CCL20. More interestingly, they also found that the changes in DC migration required neither viable *S. typhimurium* nor bacterial internalisation, since similar alterations were observed following coincubation with heat-killed *Salmonella* or when CCD was present in the infection culture (Cheminay et al., 2002).

Maturation of DC has been described as a general phenomenon observed in response to viable bacteria, or bacterial products such as LPS (Cutler et al., 2001; Svensson et al., 2000). Our earlier results have shown that *S. typhimurium* induced the maturation of BMDC by upregulation of costimulatory molecules such as CD80, CD86, CD40 and MHC class II I-A/I-E (Figure 3.16). Here, we showed that CCR7 upregulation occurred upon *S. typhimurium* induced maturation of BMDC, which served as another parameter to confirm DC maturation. Interestingly, by FACS-sorting of BMDC into immature (CD11c+ $MHCII^{low}$) and mature (CD11c+$MHCII^{high}$) populations followed by RT-PCR analysis of CCR7 gene expression, it has been shown that CCR7 is expressed on mature DC but not on immature DC (Cheminay et al., 2002). However, our uninfected BMDC displayed quite a high level of CCR7 expression as compared to uninfected BMMac at both the mRNA and protein level (Figure 5.5-5.7). This is owing to the fact that even prior to infection our BMDC culture was a heterogenous population comprising mature MHC II^{high} and immature MHC II^{low} subpopulations (Figure 3.7). *S. typhimurium* infection or LPS treatment dramatically increased the MHC II^{high} subpopulation and decreased the MHC

Il^{low} subpopulation, which correlates with the increase of CCR7 on C5 (TS) infected or LPS treated BMDC. Other studies have also confirmed that CCR7 expression on murine BMDC is an indicator of the maturity (Ritter et al., 2004). Consistent with our results, they found that about 40% of their unstimulated BMDC were positive for CCR7 mRNA, and incubation with LPS induced the maturation of all BMDC in culture. Interestingly, LPS treatment only marginally increased the number of mature CCR7+ splenic DC, indicating their more mature state (Ritter et al., 2004).

CCR7 has also been proposed to play a number of additional roles in the immune system. For example, CCR7 induces intracellular signalling that inhibits apoptosis of mature DC (Sanchez-Sanchez et al., 2004). Stimulation of CCR7 with its ligands induce activation of PI3k and prolonged activation of its downstream effector Akt1, a common signalling pathway that regulates apoptosis in a variety of cells (Strasser et al., 2000). Akt1 may then activate IκB kinase, which induces degradation of IκB. Upon IκB degradation, NFκB translocates to the nucleus and stimulates transcription from a variety of antiapoptotic genes (Datta et al., 1999; Karin and Lin, 2002). Since it has been suggested that DC terminate their life cycle in the lymph node (Kamath et al., 2002), an extended longevity, favoured by signalling from CCR7, may increase the probability of antigen presentation and stimulation of antigen-specific T cells. In this regard, our results showed both a high degree of apoptosis detected on C5 (TS) infected BMDC and an upregulation of CCR7 expression. An explanation for this discrepancy could be that an antiapoptotic effect exerted from CCR7 signalling might be overwhelmed by an apoptotic effect induced by

S. typhimurium infection. Conversely, a lack of a protective effect of CCR7 expression might have been due to the absence of the chemokine ligand.

Although both BMDC and BMMac upregulated CCR7 expression upon C5 (TS) infection or LPS treatment, uninfected BMDC had a much higher level of expression of CCR7 mRNA than uninfected BMMac (Figure 5.5). Such differences were further confirmed using a CCL19-fusion protein for detection of CCR7 at the protein level. Uninfected BMDC had a detectable level of CCR7 expression, which was greatly increased following infection (Figure 5.6). However, uninfected BMMac expressed no detectable CCR7 protein; upon infection, expression became detectable but remained low compared to BMDC (Figure 5.7). As a key chemokine receptor responsible for cellular trafficking to the SLO, the dramatic difference between the levels of CCR7 protein expressed on these two cell populations may ultimately determine their differential migration patterns *in vivo*. Conversely, CCR6 was observed to be downregulated on both cell types.

The large difference between CCR7 expression on BMDC and BMMac has been clearly demonstrated by RNase protection assay in another study (Bhatt et al., 2004). Furthermore, chemokine receptor expression on DC and macrophage has been shown previously to be modulated in a consistent way with what we found in our study (Jeannin et al., 2003). In this previous study, immature DC exposed to outer membrane protein A (a microbial product of gram-negative bacteria) gradually acquired CCR7 expression (detected at RNA and protein levels), whereas CCR1 and CCR5 expression disappeared after 24

hours and remained undetectable for up to 48 hours. In contrast, CCR1 and CCR5, but not CCR7 were constitutively expressed by macrophages. Upon stimulation, CCR7 expression was induced within 24 hours on macrophages with CCR1 and CCR5 expression down-regulated concomitantly (Jeannin et al., 2003). Taken together, these results further confirm that the "switch" of chemokine receptors on both DC and macrophages is a general phenomenon upon stimulation with microbes or their products.

Results from the chemotaxis assay demonstrated that CCR7 upregulation and CCR6 downregulation on BMDC in response to C5 (TS) infection or LPS treatment were both functional events, since after stimulation, BMDC lost the ability to migrate towards CCL20, but acquired the ability to migrate towards CCL19 (Figure 5.8-5.9). As a result, the functional responsiveness to CCL19 was greater than the responsiveness to CCL20 (20% vs up to 5%) upon C5 (TS) infection. This may be sufficient to allow infected BMDC to migrate to where their ligands are, *in vivo*, such as SLO. Although the functional migratory ability of BMMac was tested by the same chemotaxis assay used for BMDC, BMMac did not show any ability to migrate towards CCL19 (data not shown). This is likely due to several factors. Firstly, macrophages are adherent cells and attach to the surface of culture flasks in a short time (van Furth, 1992), and this feature has been used to remove macrophage from other nonadherent cells in culture (Lutz et al., 1999). Therefore, during the 4-hour incubation time in the chemotaxis assay, they might simply have attached to the bottom of polycarbonate filter. Secondly, the level of CCR7 on BMMac, even after upregulation upon stimulation, is far lower than on BMDC. This may not be

sufficient to allow BMMac migrate in response to CCL19. Also, the failure of macrophages to migrate in the chemotaxis assay might be due to another technical problem, since we used 5.0 μm pore size polycarbonate filters in the assay. This pore size is suitable for DC, but could be too small for macrophages. Jeannin and colleagues performed *in vitro* chemotaxis assays successfully on both DC and macrophages using 8.0 μm pore size filter, although they also claimed that a longer incubation period (24 hours) is required to observe macrophage migration than DC migration, which was observed only after 90 minutes exposure to CCL21 (Jeannin et al., 2003).

Potentially, three approaches can be used to block the effect of CCR7: *in vivo* neutralisation with an anti-CCR7 antibody, desensitisation using CCR7 ligands and use of CCR7 deficient mice. Due to the previous lack of a commercial mouse CCR7-specific monoclonal antibody, desensitisation of CCR7 was initially chosen to block the effect of CCR7. Since CCL19 has been shown to have a 4-fold better efficacy for desensitisation than CCL21 (Kohout et al., 2004), CCL19 was chosen to desensitise CCR7. This process of agonist specific desensitisation is called "homologous desensitisation" (Ali et al., 1999).

Since it has been suggested that homologous desensitisation requires preincubation with a high concentration of chemokines (Maghazachi, 2000), we firstly set out to choose the optimal concentration of CCL19. Based on the finding that 5 μg/ml and 10 μg/ml of CCL19 exerted comparable effects on desensitisation on C5 (TS) infected BMDC (Figure 5.10), 5 μg/ml of CCL19 was used. C5 (TS) infected BMDC migration towards CCL19 *in vitro* was greatly

inhibited by desensitisation (Figure 5.10 and Figure 5.11). CCL20 was originally chosen to serve as a control for the specificity of desensitisation. Interestingly, we found that the level of migration of desensitised BMDC (either uninfected or C5 (TS) infected) toward CCL20 or in medium alone was also reduced (Figure 5.10). This could indicate that in addition to homologous desensitisation, so called "heterologous desensitisaton" has also occurred. Another possibility is that DC themselves produce some CCL19, which is responsible for stimulating the background migration. In this case, CCL19 desensitisation would still be considered homologous.

A comparable number of cells were recovered from the PL after injection of desensitised or non-desensitised BMDC (Figure 5.12a A and Figure 5.12b A), which indicated that a comparable number of cells had been transferred into the peritoneal cavity two-days previously. In contrast, significantly fewer desensitised cells were found in MLN (Figure 5.12a B and Figure 5.12b B). This result suggested that the migration of BMDC to the regional LNs *in vivo* was dependent on the upregulated expression of CCR7 caused by *S. typhimurium* infection.

Although, the two desensitisation experiments gave similar results in MLN, the differences observed in the second experiment were not statistically significant ($p=0.05$). This might relate to the fact that smaller numbers of CFSE labelled cells were retrieved from all organs we examined in this experiment. As the numbers of labelled cells present in MLN were small, a moderate reduction in numbers may not readily show obvious significance. In addition, smaller sample

numbers (3 mice per group in this experiment in contrast to 4 mice in the first experiment) could be another factor affecting the significance of results.

Considerable variance existed between two desensitisation experiments with the respect to the effect on cell migration to the spleen. Variability may be caused firstly by extensive manipulation of DC required in the experiments. Additionally, desensitisation is a complex process that involves a diverse array of signalling molecules (Maghazachi, 2000). Therefore, differing signalling pathways could have occurred during two experiments. Since the spleen data failed to show consistent results from two desensitisation experiments, we were unable to draw any firm conclusion as to whether CCR7 was required to guide DC to the spleen.

Even in MLN, the accumulation of injected BMDC was only reduced, but not abolished by CCR7 desensitisation. This could be due to two reasons. Firstly, results from the *in vitro* chemotaxis assay had shown that CCR7 desensitisation was not able to completely block migration of CCL19 (Figure 5.11). This residual effect of CCR7 *in vitro* most likely was responsible for the residual migration to the SLO *in vivo*. Secondly, other molecules apart from CCR7 may also play a role in the directing of mature DC migration to regional lymph nodes. A role for CCR8 and other chemokine pathways in the migration of monocyte-derived DC to lymph nodes has been demonstrated recently (Qu et al., 2004). Additionally, acquisition of other receptors such as CXCR5 as DC matured enables them to migrate to chemokines BLC/CXCL13, which is constitutively produced in B cell follicles in SLO (Oppenheim et al., 2002; Wu and Hwang,

2002; Yu et al., 2002). Hence, the CXCR5-BLC/CXCL13 axis may potentially also contribute to the migration of BMDC we observed in our study.

To provide a clear answer regarding the role of CCR7 in directing migration of transferred DC, CCR7 deficient mice (Forster et al., 1999) were used. We found that migration of DC to the MLN was highly CCR7-dependent, as few if any CCR7 deficient DC were found in MLN. Reduced numbers of CCR7-deficient DC were also found in ILN, although the difference was not statistically significant, likely due to the relatively smaller number of wild type DC detected in this location. However, DC migration to the spleen seemed to be unaffected by CCR7 deficiency, as comparable numbers of CCR7 deficient and wild type DC were observed in spleen.

Surprisingly, a slight, but significant reduction in the number of CCR7 deficient cells was also observed in the PL (Figure 5.13A). This was unlikely to be due to variability in the number of injected cells, we carefully injected equal number of *S. typhimurium* infected BMDC into the peritoneal cavity of mice in each group (1×10^7/mouse). One possibility was that the efficiency of infection with C5 (TS) differed between the two groups. This could lead to differences in DC maturation state and survival. Alternatively, CCR7 itself could play a role in DC retention and/or survival in the PL.

Together with data from desensitisation experiments, results from experiments using CCR7 deficient DC clearly demonstrated that the migration to the MLN of *S. typhimurium* infected BMDC after i.p. adoptive transfer was highly dependent

on the CCR7-CCL19/CCL21 axis. These data are in agreement with previous findings that *in vitro* differentiated CCR7-deficient DC fail to migrate into the draining LN (popliteal LN) 36 hours after intracutaneous injection into the footpad of mice (Ohl et al., 2004). In contrast, CCR7 deficiency failed to inhibit the migration of DC to spleen, suggesting that migration of *S. typhimurium* infected BMDC to the spleen was not CCR7-dependent.

The inconsistent results observed in desensitisation experiments could be due to the possible occurrence of heterologous desensitisation of other chemokine receptors. Homologous desensitisation occurs in receptors in the agonist-occupied state and involves phosphorylation by G-protein-coupled receptor kinases. These phosphorylated receptors associate with members of the arrestin family of proteins resulting in a decreased affinity of the receptor for G-proteins and internalisation. Heterologous desensitisation (also called cross-desensitisation) occurs when a receptor loses its responsiveness following phosphorylation by second messenger-activated kinases (i.e. protein kinase A or protein kinase C). These kinases can be activated by different receptors or signaling processes (Chuang et al., 1996; Freedman and Lefkowitz, 1996). Heterologous desensitisation does not require agonist occupancy and does not lead to arrestin-mediated receptor internalisation (Ali et al., 1999). Since many cells, for example DC, have multiple GPCRs that signal through common effectors, it is not surprising that cross-regulation can occur in the signaling pathways from one G-protein coupled receptor to another (Hosey, 1999). As mentioned earlier, the potential occurrence of heterologous desensitisation of other chemokine receptors was indicated in Figure 5.10. Thus, after CCL19-

induced desensitisation, either uninfected or C5 (TS) infected DC showed an impaired ability to migrate to CCL20 (ligand for CCR20) compared to non-desensitised cells. Some other chemokine receptors (so far unknown) might also have been cross-desensitised in the first desensitisation experiment, directly affecting the migration of DC to the spleen.

DC, after encountering antigen in the peripheral tissues, migrate to the draining LN through afferent lymphatics, and settle in the vicinity of high endothelial venules (HEVs) in the paracortex (T cell zone) displaying antigens for lymphocytes to screen. Most lymphocytes enter the lymph nodes from the peripheral blood and further migrate into the lymph-node parenchyma through HEVs. If seen and activated by antigen, lymphocytes proliferate and undergo profound differentiation (Miyasaka and Tanaka, 2004). As mentioned earlier, and in more detail in another report, CCL21 is expressed on HEVs and CCL19 is produced by stromal cells in the T cell zone surrounding HEVs. Therefore, both are responsible for bringing mature DC and naive lymphocytes together in SLO through their CCR7 expression (Dieu et al., 1998; Ngo et al., 1998) (Miyasaka and Tanaka, 2004). CCR7 deficiency on DC hence would severely affect this migratory process of DC, as we found in our study.

DC access the spleen initially via the blood. Migration to the lymphoid regions (white pulps) of spleen then occurs through the interaction of CCR7 with CCL19 and CCL21. However, another function of the spleen is to filter the effete cells, debris and microorganisms that have entered the blood stream. This function is exerted primarily in the red pulp of spleen. Since the DC we transferred had

reached their final stage of development (*in vitro* cultured for 7 days followed with infection with *Salmonella*), the ultimate fate of these transferred cells is death and they will be cleared by the host eventually. Once they access the blood, it is likely that they would be trapped in the red pulp of the spleen and be degraded as effete or unhealthy cells. Thus, initial entry of DC into the spleen may not require the involvement of CCR7. Whether the CCR7-deficient DC enter the white pulp is an interesting question for further study. Nevertheless, even if they cannot migrate autonomously to the white pulp, entering the red pulp DC could still be able to play a role in antigen presentation through the participation of endogenous DC; a pathway which has been suggested in another study of anti-*Salmonella* immunity (Yrlid and Wick, 2000) and also implicated in our immunohistochemical staining of the spleens of mice receiving adoptively transferred cells (Figure 4.9 and Figure 4.10).

5.4 Conclusion

Based on the differential migration pattern of DC and macrophages observed in chapter 4, we set out to explore the potential mechanisms behind these results. Apoptotic cell death was first evaluated on BMDC and BMMac after C5 (TS) infection. BMMac did not show a greater susceptibility to apoptosis than BMDC, which excluded the possibility that the low recovery of BMMac *in vivo* was due to a greater extent of cell death after C5 (TS) infection. Secondly, changes in expression of CCR7 and CCR6 upon *S. typhimurium* infection were analysed for both types of cells. CCR6 was greatly downregulated on BMDC in contrast to a slight decrease on BMMac upon infection. CCR7 was highly expressed on uninfected BMDC but absent on uninfected BMMac at the protein level, and its expression was highly increased on C5 (TS) infected BMDC and moderately increased on C5 (TS) infected BMMac at either the mRNA or protein levels. Although expression followed a similar pattern with these two chemokine receptors on the two cell types, differences in the absolute level of expression upon *S. typhimurium* infection may be responsible for the differential migration profiles exhibited *in vivo*. Finally, in order to determine whether CCR7 upregulation was playing a major role in the specific migration of BMDC to the SLO upon *S. typhimurium* infection. Two approaches were taken. Initially, *in vitro* CCR7 desensitisation was performed prior to adoptive transfer of infected BMDC. Variability in the results of these experiments meant that it was difficult to draw conclusions regarding the role of CCR7 in migration to the spleen, although, the migration of infected DC to the MLN was consistently diminished by *in vitro* desensitisation of CCR7. Subsequently, experiments using CCR7 deficient BMDC clearly showed migration of *S. typhimurium*-infected BMDC to

the MLN was mediated by a CCR7-dependent mechanism, whereas migration of same cells to the spleen was not regulated by CCR7.

Chapter 6: Antigen presentation ability of BMDC and BMMac upon recombinant *S. typhimurium* infection

6.1 Introduction

The immune system has evolved two lines of defense to protect against harmful microorganisms such as *S. typhimurium*. The first line of defense, the innate immune system, includes physical barriers such as skin, mucous membranes, and non-specific engulfment of invading microorganisms by phagocytic cells such as macrophages, immature DC and polymorphonuclear neutrophils. An array of elimination mechanisms generated by these phagocytic cells includes secretion of inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12 and IL-18 and production of oxygen and nitrogen radicals such as NO (Wick, 2004). However, in most cases of *Salmonella* infection, the innate immune response fails to eliminate the bacteria from the host. Under such circumstances, the second line of defense, the acquired immune system, with T and B lymphocytes as key components, is required for eradication of bacteria and protection against re-infection with the same pathogen (Mittrucker et al., 2000). The innate immune system is linked to the adaptive immune system through a group of cells collectively called APCs including DC, macrophages and B cells. These cells function to take up bacterial antigens, process the proteins into peptide fragments and present the peptides to T lymphocytes (CD4+ and CD8+) in the context of major histocompatibility complex (MHC) molecules. DC and macrophages could both play a part as phagocytes to control invasion of

Salmonella in the innate immune response. In addition they could both act as APC to induce specific immune responses to combat the bacterial infection.

In this chapter, we set out to look into the antigen presentation capacity of DC and macrophages upon bacterial infection, and whether this capacity differs between these two types of APCs, with respect to their ability to present antigen to either memory T cells or naive T cells. Finally, the ability of adoptively transferred *S. typhimurium* infected BMDC migrating to SLO to present *Salmonella* antigen to naive T cells was investigated using a co-adoptive transfer system.

6.2 Results

6.2.1 In vitro system

6.2.1.1 Comparison of the ability of BMDC and BMMac to stimulate previously stimulated T cells

The response of previously activated T cells to antigen presented by *S. typhimurium* infected BMDC and BMMac was determined *in vitro* using the IFN-γ ELISpot assay. CTL specific for NP_{383} and BG_{876} were used in this experiment, and the generation of these two T cell lines is described in the materials and methods. The NP_{383}CTL responds to the HLA-B27 restricted epitope 383-391 from influenza A virus nuclear protein, and BG_{876}CTL responds to the H-Kd restricted epitope 876-884 from β-galactosidase. These CTLs had been generated by a primary stimulation *in vivo* followed by *in vitro* stimulations and so represented a population of antigen experienced T cells. The CTLs were used to assess *Salmonella* specific responses by using recombinant *Salmonella* expressing these T cell epitopes, as described in materials and methods. BMDC and BMMac were infected with recombinant *Salmonella,* co-cultured with the T cell lines and IFN-γ production measured as an indication of a T cell response. IFN-γ ELISpot assays showed that both BMDC and BMMac were able to stimulate NP_{383} cytotoxic (Figure 6.1A) and BG_{876} T cell responses (Figure 6.1B) after peptide pulsing or infection with recombinant SL3261. In addition, this stimulation was antigen specific since infection with recombinant SL3261 expressing the irrelevant peptide failed to induce IFN-γ production from the antigen specific T cells. Notably, peptide- pulsed BMDC and BMMac

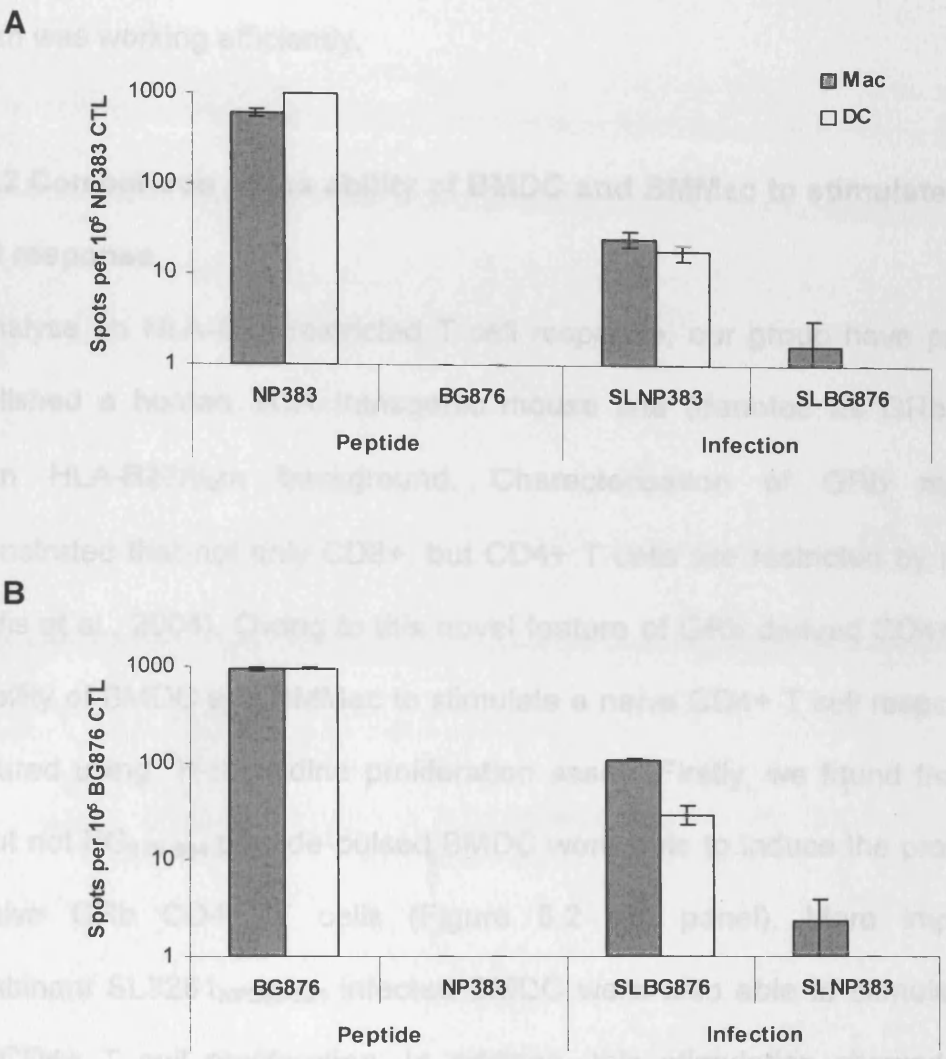


Figure 6.1 Analysis of T cells responses *in vitro* induced by recombinant *S. typhimurium* SL3261 infected DC and Mac using IFN-γ ELISpot assay. Specific CTL lines were prepared and 10^5 T cells were seeded into wells of 96-well plates. In parallel, DC or Mac were either pulsed with 1 μM peptide or infected with recombinant SL3261 as indicated and 10^4 cells were added to each well. After 48 hours incubation, T cell responses were measured by production of IFN-γ, which was detected by IFN-γ ELISpot. Spots formed by IFN-γ secreting cells were counted and results expressed as mean values ± SD of triplicates performed in assay. (A) $NP_{383-391}$ CTL line response. (B) $BG_{876-884}$ CTL line response. Results are representative of 2 experiments.

showed a strong ability to stimulate CTL responses indicating that the testing system was working efficiently.

6.2.1.2 Comparison of the ability of BMDC and BMMac to stimulate a naive T cell response

To analyse an HLA-B27 restricted T cell response, our group have previously established a human TCR transgenic mouse line (denoted as GRb) on the human HLA-B27/β_2m background. Characterisation of GRb mice has demonstrated that not only CD8+, but CD4+ T cells are restricted by HLA-B27 (Roddis et al., 2004). Owing to this novel feature of GRb derived CD4+ T cells, the ability of BMDC and BMMac to stimulate a naive CD4+ T cell response was measured using ^{3}H-thymidine proliferation assay. Firstly, we found that $NP_{383-391}$, but not $BG_{876-884}$ peptide-pulsed BMDC were able to induce the proliferation of naive GRb CD4+ T cells (Figure 6.2 top panel). More importantly, recombinant $SL3261_{NP383-391}$ infected BMDC were also able to stimulate naive GRb CD4+ T cell proliferation. In addition, this stimulation showed antigen specificity as no response was observed with BMDC infected with recombinant $SL3261_{BG876-884}$ (ie, expressing an irrelevant peptide) (Figure 6.2 bottom panel). In contrast, neither peptide pulsed (Figure 6.2 top panel) nor bacteria infected BMMac (Figure 6.2 bottom panel) were able to induce a response by naive GRb CD4+ T cells.

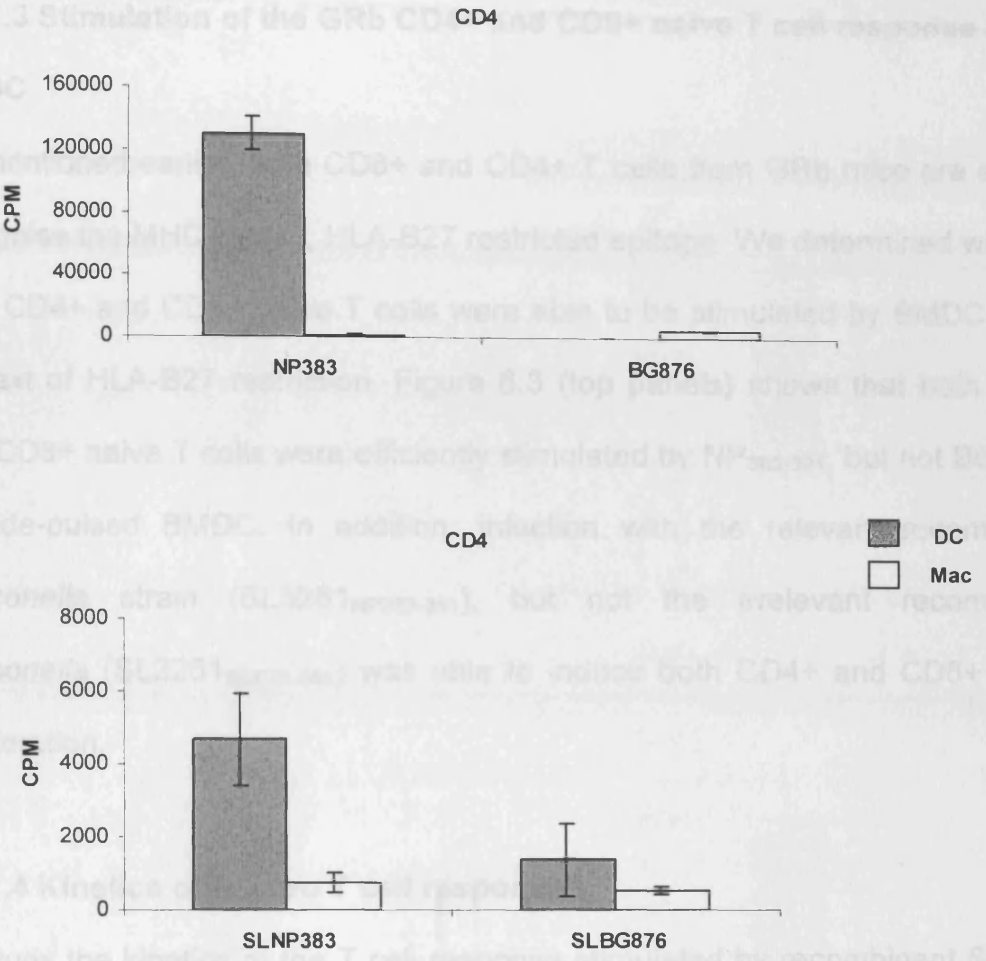


Figure 6.2 *In vitro* naïve T cell responses induced by recombinant SL3261 infected BMDC and BMMac. Naive CD4 T cells were purified from spleens and lymph nodes of GRb transgenic mice by negative selection. CD4 (5×10^4) cells were seeded into the wells of 96-well plates. In parallel, BMDC and BMMac (2×10^4) were infected with recombinant *S. typhimurium* SL3261 (lower panel) or pulsed with peptide (used as controls) (upper panel) as indicated. After 4 days incubation, the plates were pulsed with ^{3}H-thymidine for 16 hours. Data are expressed as the mean values ± SD of triplicates performed in assay. Results represent two experiments.

6.2.1.3 Stimulation of the GRb CD4+ and CD8+ naive T cell response by BMDC

As mentioned earlier, both CD8+ and CD4+ T cells from GRb mice are able to recognise the MHC class I, HLA-B27 restricted epitope. We determined whether both CD4+ and CD8+ naive T cells were able to be stimulated by BMDC in the context of HLA-B27 restriction. Figure 6.3 (top panels) shows that both CD4+ and CD8+ naive T cells were efficiently stimulated by $NP_{383-391}$, but not $BG_{876-884}$ peptide-pulsed BMDC. In addition, infection with the relevant recombinant *Salmonella* strain ($SL3261_{NP383-391}$), but not the irrelevant recombinant *Salmonella* ($SL3261_{BG876-884}$) was able to induce both CD4+ and CD8+ T cell proliferation.

6.2.1.4 Kinetics of *in vitro* T cell response

To study the kinetics of the T cell response stimulated by recombinant SL3261 infected BMDC, T cell responses were assessed over 4 days after stimulation using CFSE labelling. CFSE fluoresence is split between daughter cells as cells divide, so we can measure proliferation by a loss in CFSE density. Figure 6.4 A and B show typical profiles of responding and non-responding T cells after stimulation. Figure 6.4 C and D show several points of information. Firstly, the antigen specificity of the response was apparent, as a high percentage of CFSE loss was observed when BMDC were pulsed with $NP_{383-391}$ peptide or infected with recombinant Salmonella expressing the relevant antigen, but not when exposed to irrelevant antigen ($NP_{366-374}$). Secondly, the efficiency of antigen stimulation recombinant *Salmonella*-infected BMDC was comparable to that of peptide pulsed ones. Thirdly, the stimulated T cell response was observed from

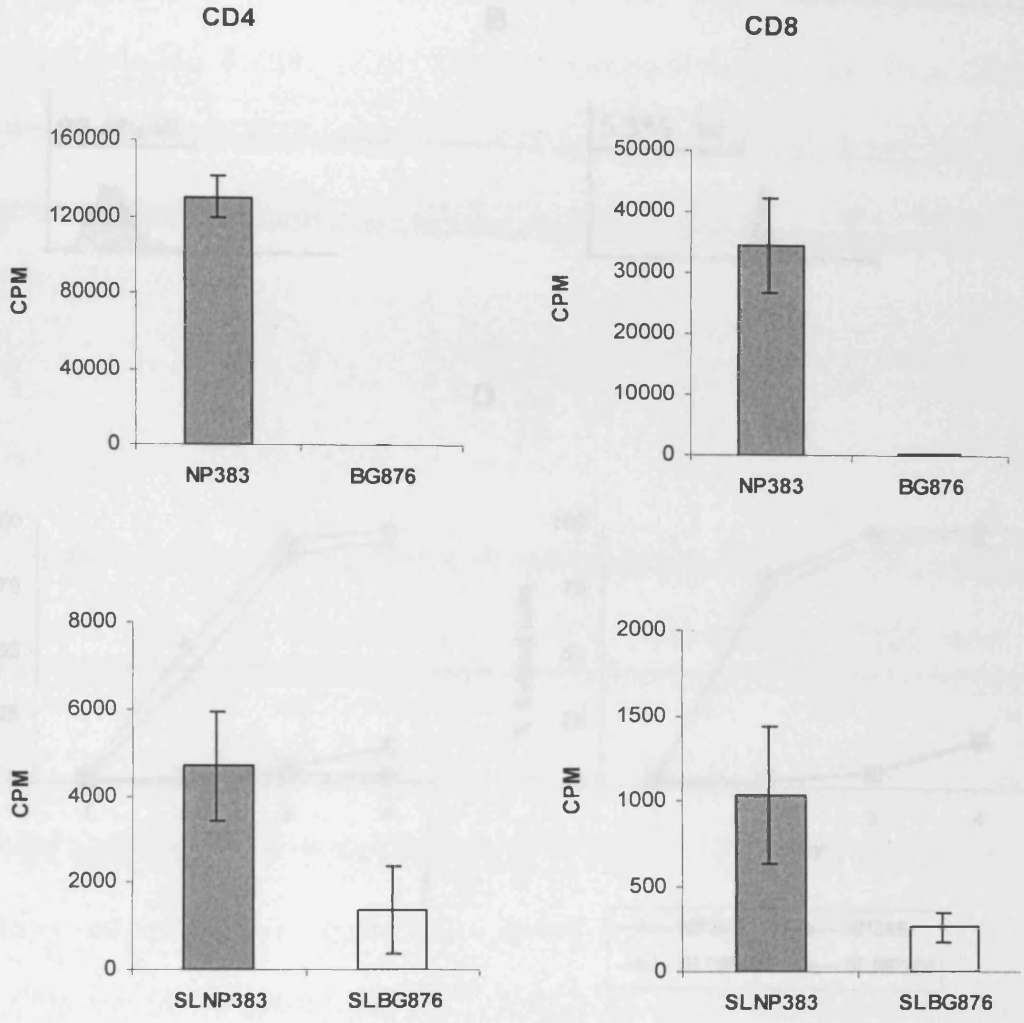


Figure 6.3 *In vitro* naïve T cell responses induced by recombinant SL3261 infected DC. Naive CD4 (left panel) or CD8 (right panel) T cells were purified from spleens and lymph nodes of GRb transgenic mice by negative selection. CD4 (5×10^4) or CD8 (3×10^4) cells were seeded into the wells of 96-well plates. In parallel, BMDC were infected with recombinant *S. typhimurium* SL3261 (lower panel) or pulsed with peptide (used as controls) (upper panel) as indicated. After 4 days incubation, the plates were pulsed with ^{3}H-thymidine for 16 hours. Data are expressed as the mean values ± SD of triplicates performed in assay. Results represent two experiments.

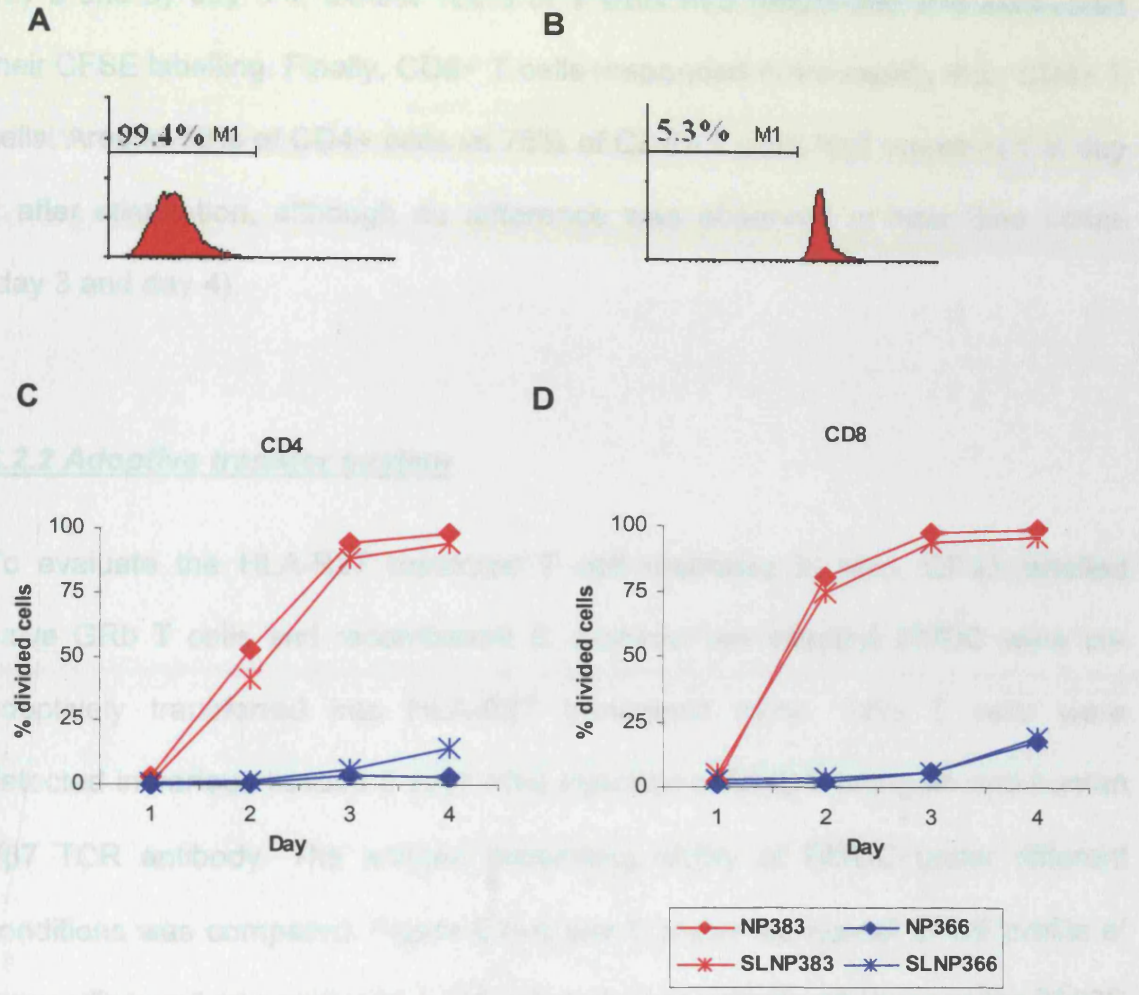


Figure 6.4 Time course of *in vitro* T cell responses stimulated by recombinant SL3261 infected DC. CD4 or CD8 T cells were prepared from the spleens of GRb transgenic mice by negative selection. T cells were labelled with CFSE, and then 10^5 cells were seeded into the wells of 96-well plates. In parallel, BMDC (10^4) were either infected with recombinant *S. typhimurium* SL3261 or pulsed with 1 μM peptide as indicated. The cells were harvested at the indicated time points and stained with anti-CD4 antibody (Cy-chrome) and anti-CD8 antibody (APC conjugate). The proliferation of T cell subsets is expressed as the percentage of cells that have divided at least once as assessed by flow cytometry. (A, B) Typical CFSE profiles of how the percentage was derived. (C, D) CD4 or CD8 T cell responses as indicated. Results are the representative of 2 experiments.

day 2 and by day 3-4, almost 100% of T cells had responded and exhausted their CFSE labelling. Finally, CD8+ T cells responded more rapidly than CD4+ T cells. Around 50% of CD4+ cells vs 75% of CD8+ T cells had responded at day 2 after stimulation, although no difference was observed at later time points (day 3 and day 4).

6.2.2 Adoptive transfer system

To evaluate the HLA-B27 restricted T cell response *in vivo*, CFSE-labelled naive GRb T cells and recombinant *S. typhimurium* infected BMDC were co-adoptively transferred into HLA-B27 transgenic mice. GRb T cells were detected in various tissues 3 days after injection of BMDC using an anti-human Vβ7 TCR antibody. The antigen presenting ability of BMDC under different conditions was compared. Figure 6.5 A and B show the typical CFSE profile of responding and non-responding GRb T cells respectively. Mice receiving BMDC pulsed with relevant or irrelevant peptide were used as positive and negative controls respectively. In MLN (Figure 6.5B), nearly 80% compared to 40% of transferred CD8+ T cells were CFSE low after co-transfer with BMDC infected with $SL3261_{NP383-391}$ or $SL3261_{NP366-374}$ respectively. Among CD4+ T cells, more than 40% when injected with $SL3261_{NP383-391}$-infected BMDC compared to about 20% when transferred with BMDC infected with control recombinant *Salmonella*. A similar effect was also observed in ILN. More than 60% of CD8+ and 40% of CD4+ T cells were stimulated by the co-transfer of $SL3261_{NP383-391}$ infected BMDC whereas the antigen non-specific response was less than 10% for both CD4+ and CD8+ T cells. Interestingly, although the antigen specific T

cell response was similar in both tissues, BMDC infected with the irrelevant recombinant bacteria induced a higher non-specific T cell response in MLN (40% CD8+ and 20% CD4+) than in ILN (both less than 10%).

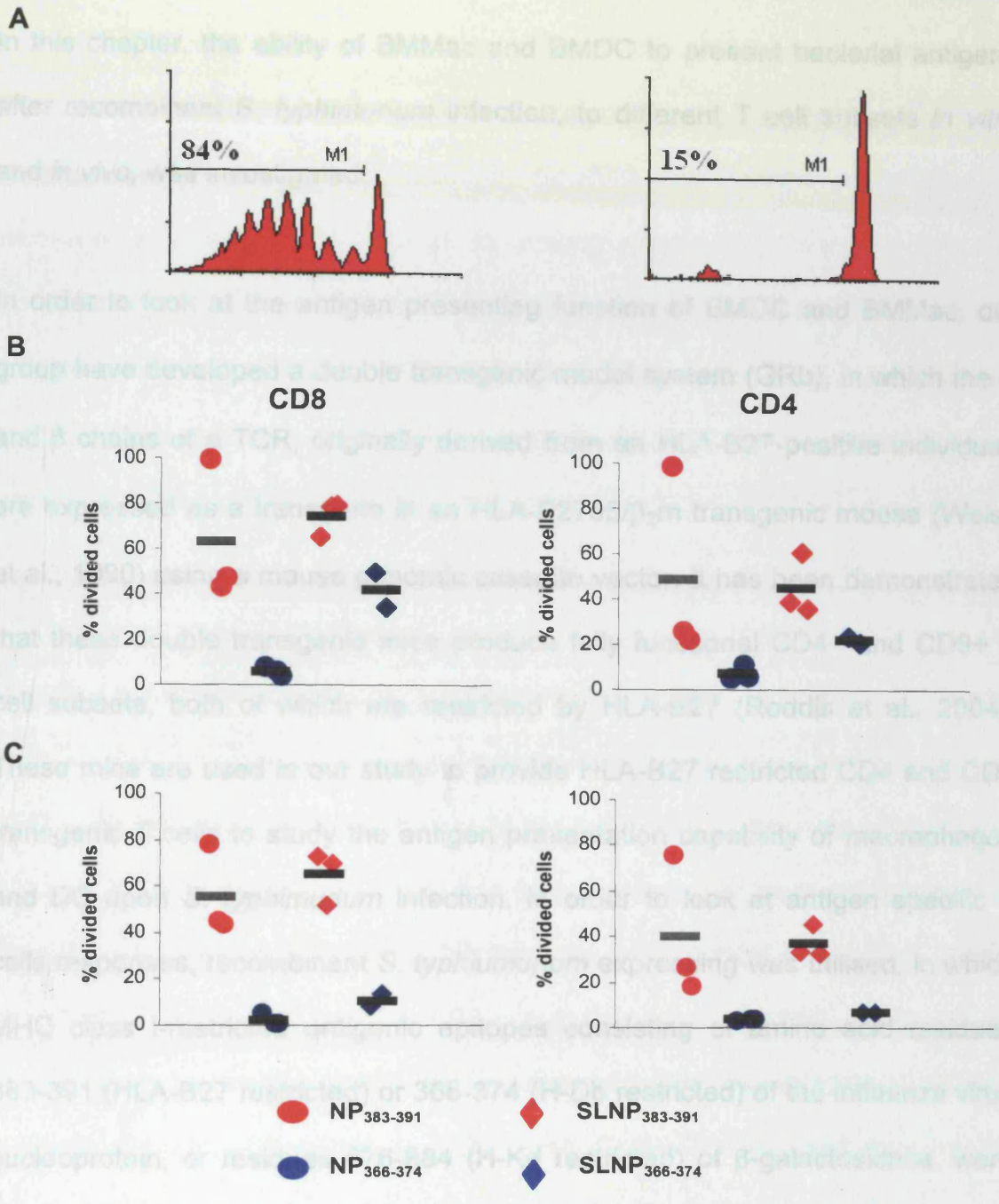


Figure 6.5 Analysis of *in vivo* T cell response by co-adoptive transfer of GRb T cells and recombinant *S. typhimurium* infected BMDC. 10^6 T cells isolated from GRb transgenic mice were adoptively transferred into HLA-B27 transgenic mice by intravenous injection after CFSE labelling. On the following day, 5×10^6 recombinant SL3261 infected or peptide pulsed BMDC as indicated were harvested and intraperitoneally injected into these mice. On day 3 after DC transfer, cells were isolated from mesenteric lymph nodes (MLN) and inguinal lymph nodes (ILN) and stained with anti-CD8 (APC), anti-CD4 (cychrome) and anti-human Vβ7 Ab (PE), the latter recognising transferred GRb T cells. The stained cells were analysed by flow cytometry and the T cell response was assessed by the percentage of cells that have divided at least once. Bars represent the mean value of 3 mice of each group part from 2 for the $NP_{366-374}$ group. (A) Typical profile of how the percentage of CFSE fluorescence loss was derived. (B, C) GRb T cell responses in MLN and ILN respectively. Results are representative of 2 experiments.

6.3 Discussion

In this chapter, the ability of BMMac and BMDC to present bacterial antigens after recombinant *S. typhimurium* infection, to different T cell subsets *in vitro* and *in vivo,* was investigated.

In order to look at the antigen presenting function of BMDC and BMMac, our group have developed a double transgenic model system (GRb), in which the α and β chains of a TCR, originally derived from an HLA-B27-positive individual, are expressed as a transgene in an HLA-B2705/β_2m transgenic mouse (Weiss et al., 1990) using a mouse genomic cassette vector. It has been demonstrated that these double transgenic mice produce fully functional CD4+ and CD8+ T cell subsets, both of which are restricted by HLA-B27 (Roddis et al., 2004). These mice are used in our study to provide HLA-B27 restricted CD4 and CD8 transgenic T cells to study the antigen presentation capability of macrophages and DC upon *S. typhimurium* infection. In order to look at antigen specific T cells responses, recombinant *S. typhiumurium* expressing was utilised, in which MHC class I-restricted antigenic epitopes consisting of amino acid residues 383-391 (HLA-B27 restricted) or 366-374 (H-Db restricted) of the influenza virus nucleoprotein, or residues 876-884 (H-Kd restricted) of β-galactosidase, were expressed as fusion proteins with the type III secreted protein Sop E (Wood et al., 1996) using *S. typhimurium* strain SL3261. This recombinant system, together with the T cells derived from GRb transgenic mice, enabled us to study HLA-B27-restricted T cell responses resulting from recombinant *S. typhimurium* infection.

Although recombinant *Salmonella* are both available for strain SL3261 and C5 (TS), C5 (TS) was not used for experiments in this chapter because previous work by my colleague has suggested that antigen presentation was more efficient after infection with SL3261 than C5 (TS). The possible explanation for the lower efficiency of C5 (TS) in an antigen presentation assay is that this strain optimally grows at 30°C (Hormaeche et al., 1981), at this temperature, the efficiency of the *Salmonella* TTSS, by which *Salmonella* deliver their effector proteins such as SopE into host cells, might be affected resulting in less antigen (epitope) delivered through the fusion protein.

Macrophages and DC both play an important role in the bridging the innate and adaptive immune responses to *S. typhimurium* infection. This is manifested by their sentinel function in capturing bacteria coupled with their ability to act as APC. Although these two types of cells are both able to degrade bacteria and process bacterial proteins into peptide fragments, it has been suggested that the pathways they use to accomplish this may not identical (Yrlid et al., 2000). In addition, according to their different regulation of MHC class II and costimulatory molecules upon *S. typhimurium* infection, their capacity to present processed bacterial peptides to CD4+ and CD8+ T cells, and therefore to initiate an effective immune response against this bacterium, may differ.

Due to the association of reactive arthritis with the MHC I molecule, HLA-B27, and the normal function of HLA-B27 to present peptides for recognition by CD8+ T cells, the ability of BMDC and BMMac to present peptides to CD8+ T cells after *S. tyhphimurium* infection was firstly compared using an IFN-γ

ELISpot assay. We showed that recombinant *S. typhimurium* infection rendered both BMDC and BMMac able to efficiently induce a response by CTL T cell lines against either $NP_{383-391}$ or $BG_{876-884}$ in an antigen specific manner (Figure 6.1 A and B). The reason why bacterial infection could result in a CD8+ T cell response in our study is worthy of discussion here. Typically, "exogenous" antigens such as bacteria are internalised by APC and processed within phagolysosomal compartments, producing peptides that are loaded onto MHC-II molecules in compartments of the endosomal system and transported to the cell surface for recognition by CD4+ T cells (Wolf and Ploegh, 1995). In contrast, "endogenous" antigens are synthesised within the presenting cells and processed within the cytosol by the proteasome, and produce peptides that are transported into the endoplasmic reticulum by the TAP. Peptides are then loaded onto MHC-I molecules in the endoplasmic reticulum, which then transported to the cell surface and presented to CD8+ cytotoxic T cells (Heemels and Ploegh, 1995). However, numerous studies have suggested that these two pathways are not completely separated (Jondal et al., 1996; Lechler et al., 1996; Rock, 1996). For instance, exogenous antigens can also enter the conventional class I processing pathway by penetration from vesicular compartment into the cytosol, as achieved by some microbes (Brunt et al., 1990; Yewdell et al., 1988).

Since *S. typhimurium* is initially extracellular upon entering a host but can ultimately reside inside host cells and grow as an intracellular bacterium, it has been reported that two pathways of antigen processing may potentially be involved in antigen presentation from *S. typhimurium* (Svensson et al., 1997a).

S. typhimurium has evolved a system termed the TTSS to deliver bacterial effector proteins such as SopE from the bacterial cytoplasm into the host cell cytosol using a supramolecular structure called the needle complex (Galan and Collmer, 1999). In our study, recombinant *S. typhimurium* was prepared based on this mechanism, which enabled expression of MHC I restricted T cell epitopes ($NP_{383-391}$ or $BG_{876-884}$) as fusion proteins with SopE in the cytosol of infected APC during *S. typhimurium* infection. In this respect, our results are in agreement with previous studies that both BMMac and BMDC are efficient at processing *S. typhimurium* derived peptides for MHC I presentation using a Crl-OVA expressing system (Svensson et al., 1997a; Yrlid et al., 2000).

CD8+ T cells contribute to the protective immune response to *Salmonella*, since β_2m-deficient mice were shown to be more susceptible to *Salmonella* infection (Lo et al., 1999) (Mastroeni et al., 1992) (Nauciel, 1990). Nevertheless, it is clear that CD4+ T cells play a more important role in immunity to *Salmonella*, particularly from adoptive transfer experiments (Mastroeni et al., 1992) (Nauciel, 1990). In addition, in the case of HLA-B27 associated diseases, adoptive transfer studies showed that CD4+ T cells were more efficient in transferring inflammatory disease from disease-prone HLA-B27+ transgenic rats to HLA-B27 nude rats (Breban et al., 1996). However, in these circumstances, MHC class II molecules, which are conventionally recognised by CD4+ T cells, were not required for disease development because MHC II-negative, HLA-B27+ transgenic mice still developed spontaneous disease (Khare et al., 1998). In view of the identification of HLA-B27-reactive human CD4+ T cells from HLA-B27+ SpA patients (Boyle et al., 2001), this unconventional recognition pattern

of HLA-B27 specific CD4+ T cells has been suggested to contribute to the pathogenesis of SpA (Boyle and Hill Gaston, 2003).

Our group has established a double transgenic mouse model (GRb) that can be used to test this notion. As mentioned earlier, the CD4 T cells in GRb transgenic mouse, specifically respond to the HLA-B27 restricted peptide $NP_{383-391}$ with a similar efficiency to CD8+ T cells (Roddis et al., 2004). We used CD4+ T cells from GRb mice to analyse the naive T cell response induced by HLA-B27 specific $NP_{383-391}$ recombinant *S. typhimurium* ($SL3261_{NP383}$) infected APC. Notably, we found that only BMDC, but not BMMac, were able to stimulate naive CD4+ T cells response upon recombinant $SL3261_{NP383}$ infection; this presentation was in an antigen specific manner as control peptide recombinant *S. typhimurium* ($SL3261_{BG876}$) infected BMDC failed to do so (Figure 6.2).

This discrepancy between these two types of cells in their ability to stimulate naïve T cells has been reported in a previous *in vitro* study (Yrlid et al., 2001b). The failure of macrophages to stimulate naive T cells *in vitro* led us to study of antigen presentation *in vivo* only using DC. The results (which will be further discussed later) were in agreement with another study showing that DC were indeed able to stimulate naive T cells after adoptive transfer *in vivo* (Yrlid et al., 2001a). More recently, it was reported that macrophages as well as DC present *Salmonella*-derived antigen to naïve or memory CD4+, but that DC are much more efficient than macrophages for stimulating CD4+ T cells (Kalupahana et al., 2005). All these observations suggest that DC have superior antigen presenting ability to macrophages, especially with respect to presentation of

antigen to naive T cells. Two reasons suggested by our study could be responsible for this: (1) Insufficient surface expression of MHC and costimulatory molecules needed to prime naive T cells on BMMac compared to BMDC after *S. typhimurium* infection. After infection with SL3261, BMMac in most cases failed to upregulate costimulatory molecules such as CD80, CD86; MHC class II molecules were poorly induced in all infections. In contrast, BMDC greatly increased the expression of all these molecules after *in vitro* infection (data not shown). (2) Upon *S. typhimurium* infection, BMDC but not BMMac migrated efficiently to SLO, where naive T cells circulate. Positioning in appropriate sites is a prerequisite for antigen-bearing DC to encounter and subsequently prime naive T cells. These two factors together with unique expression of several other molecules on DC (including OX40-L and 4-1BB) (Banchereau et al., 2000) contribute to the superior capacity of DC to stimulate naive T cells.

Both CD4+ and CD8+ were able to respond to HLA-B27 restricted peptide presented by BMDC after recombinant bacteria infection (Figure 6.3). The ability of immature BMDC to phagocytose and present *Salmonella*-derived antigens to CD8+ and CD4+ T cells has been reported (Svensson et al., 1997b). In that study, the mouse MHC I (Kb)-restricted 257-264 epitope from chicken egg OVA and the MHC II (I-Ak)-restricted 46-61 epitope from hen egg white lysozyme were expressed as fusion proteins in the cytoplasm of *S. typhimurium* as CrI-OVA and CrI-HEL fusion proteins, and antigen processing and presentation was analysed using T cell hybridomas. The presentation of *Salmonella* antigen to CD4+ in their study was by MHC II molecules, whereas in

our study, CD4+ T cells were primed by the MHC I molecule HLA-B27. Another study, using a similar antigen model system (CrI-OVA) in *E. coli*, demonstrated that MHC I presentation to CD8+ T cells occurred by a cytosolic MHC-I presentation pathway, which required the TAP and could be abrogated by the proteasome inhibitor MG132 (Svensson and Wick, 1999).

In order to test the *in vivo* antigen presentation ability of BMDC loaded with *S. typhimurium* derived antigen *in vitro*, CFSE labelling was chosen to monitor the T cells response. For this purpose, an *in vitro* assay was performed firstly to determine the best time point at which to analyse T cell response. Data from the *in vitro* CFSE proliferation assay clearly demonstrated that naive GRb T cells were primed by $SL3261_{NP383}$ (HLA-B27 restricted)-infected, but not antigen non-specific $SL3261_{NP366}$ (H-2Db restricted)-infected BMDC, suggesting the antigen specificity of T cell response. In addition, the results indicated that the efficiency of presentation was comparable between peptide pulsed BMDC and recombinant *S. typhimurium* infected BMDC. This suggested that by two-hours post-infection, BMDC had phagocytosed and processed *S. typhimurium* derived antigen in an efficient way, reflecting their antigen processing and presentation ability. The T cell response was observed from day 2 after mixture of T cells and BMDC. At day 3, almost all CD4+ and CD8+ had responded and exhausted their CFSE fluorescence, but the antigen non-specific response remained at a very low level. By day 4, no further T cell response could be detected (since most cells were already $CFSE^-$), but T cells started to exhibit some level of antigen non-specific response. For this reason, day 3 appeared to be the

optimal time point for observing the antigen specific T cell response under these conditions, and was chosen to be used as the time point for the *in vivo* assay.

Interestingly, in the same experiment we also found that naive CD8+ T cells responded more rapidly than CD4+ T cells. At day 2, 75% of CD8 compared to 50% of CD4 T cells had been activated and divided. The reason for the slightly more rapid activation of CD8+ than CD4+ T cells is unknown, but it could be related to recognition on HLA-B27. At present, we do not know the requirements for HLA-B27 presentation to CD4+ T cells. Data from our group reveal that despite the requirement for MHC II expression for the development of a substantial CD4+ population, the CD4+ GRb T cell response can be generated in the absence of mouse MHC II (Roddis et al., 2004). Although recognition of an abnormally folded HLA-B27 complex, such as a homodimeric structure of HLA-B27, has been suggested to explain this unconventional recognition (Boyle and Hill Gaston, 2003), whether these aberrant structures of HLA-B27 are able to present antigenic peptide to CD4+ T cells is unknown. HLA-B27 lacking associated β_2m is prone to misfold and form those aberrant structures (Luthra-Guptasarma and Singh, 2004). However, in our study, HLA-B27 was expressed together with human β_2m in our HLA-B27 transgenic mice. In another study of HLA-B27 transgenic mice it was clearly shown that cells in these mice display expression of free heavy chains of HLA-B27 on cell surface. Furthermore, *in vivo* treatment with anti-HC-specific antibody delays the development of spontaneous arthritis (Khare et al., 1996). Therefore, even in the presence of human β_2m, heavy chains of HLA-B27 are still able to form a homodimeric structures. More recently, Kollnberger and colleagues further

showed that HLA-B27 heavy chain homodimers were ligands for paired Ig-like receptors (Kollnberger et al., 2004), providing a possible explanation for the pathogenesis of HLA-B27 associated spondyloarthritis. Whether this particular binding also occurs between HLA-B27 and CD4+ T cells in our system requires further investigation.

We next tested the ability of *S. typhimurium*-infected BMDC to stimulate naive GRb T cells *in vivo* by adoptive transfer of CFSE labelled GRb T cells followed by recombinant *S. typhimurium* infected BMDC into HLA-B27 recipient mice. Specific CD8 and CD4 T cell responses were elicited in MLN and ILN of recipient mice. Interestingly, except for one mouse given peptide (NP_{383})-pulsed BMDC, the other two mice in this group showed a lower T cell response than mice given $SL3261_{NP383}$ infected BMDC. This contrasted with results from *in vitro* assay, which consistently showed that peptide pulsed BMDC elicited a better response than bacteria infected BMDC. The *in vitro* results may reflect that bacterial infected BMDC need to process the bacterial antigen to obtain peptide, likely generating less peptide than used to pulse DC, in addition to the negative effect on antigen presentation of intracellular *Salmonella* (Cheminay et al., 2005). However, for *in vivo* presentation, cell contact between DC and T cells would be dependent on DC migrating to SLO after adoptive transfer, which requires CCR7 upreguation. In this circumstance, bacterial infected DC likely had much higher expression of CCR7 than peptide-pulsed DC, since uninfected DC had only a low level of CCR7 expression (reflected in Figure 5.3). CCR7 upregulation would drive more infected BMDC to SLO such as MLN than peptide pulsed BMDC, which would facilitate *in vivo* DC and T cell interaction. In

addition, infected DC will express higher levels of co-stimulatory molecules than uninfected, peptide-pulsed DC. These factors could counter the higher level of peptide-MHC on peptide-pulsed DC, and result in a greater T cell response being observed in mice given infected BMDC than the mice given peptide pulsed BMDC.

Notably, a slightly higher non-specific response from mice receiving BMDC infected with $SL3261_{NP366}$ was observed in MLN than in ILN. This could reflect the greater numbers of BMDC that reach MLN as opposed to ILN. Although we attempted to analyse the T cell response in the spleen, visualisation of GRb T cells in this location was difficult due to the presence of strongly auto fluorescent cells, which masked the intermediate staining of the human Vβ7 antibody.

The ability for *S. typhimurium* loaded BMDC to generate a bacteria-specific T cell response in recipient mice has been reported in a previous study (Yrlid et al., 2001a), but there are several differences between our and their studies. (1) They used *S. typhimurium* expressing OVA as a model antigen, whereas we used *S. typhimurium* expressing $NP_{383-391}$ peptide. (2) Mice in their study were given DC infected with *S. typhimurium* expressing OVA on two occasions a week apart. Only one injection of *S. typhimurium* infected BMDC was given to mice in our study. (3) They analysed the T cell response two weeks after the last administration, whereas day 3 after DC injection was used in our study. (4) To detect the response, they isolated splenocytes from immunised mice and restimulated them for 24 hours with DC infected with heat-killed *S. typhimurium*

expressing OVA; whereas, adoptive transfer of naive T cells from GRb mice and no further *ex vivo* stimulation was performed in our study. (5) They analysed T cell response by intracellular IFN-γ staining, while, in our study, CFSE labelling was used. In our study, some functional tests of our T cell response, including IFN-γ and granzyme B staining, were attempted without success due to a technical problem.

Taken together, our study combined with the previous study (Yrlid et al., 2001a) demonstrated that BMDC infected with *S. typhimurium in vitro* were able to efficiently stimulate a T cell response. Our model, in which *S. typhimurium*-infected BMDC generated from HLA-B27 transgenic mice were able to present MHC I antigen to naive CD4 T cells from GRb mice, could be used as a tool to further investigate the antigen processing and presentation pathway involved in this unusual recognition with respect to their potential role in spondyloarthropathies such as ReA.

6.4 Conclusion

In this chapter, a differential antigen presentation ability of BMDC and BMMac was demonstrated using a GRb transgenic mouse model. After *S. typhimurium* infection, BMDC and BMMac both efficiently presented bacterial-derived antigen to specific antigen experienced cytotoxic T cells, whereas, only BMDC were able to activate GRb naive T cells. In addition, it was shown that BMDC infected with *S. typhimurium in vitro* could prime GRb naive T cells *in vivo*. Exploring the unique feature of our GRb mice in which CD4+ T cells are also restricted by the MHC I molecule HLA-B27, we showed that both CD8+ and CD4+ T cells are efficiently stimulated by adoptive transfer of *S. typhimurium*-infected, HLA-B27+ BMDC. The unusual recognition of MHC I-restricted peptide by CD4+ T cells makes this model of potential use for deciphering the pathogenesis of HLA-B27 associated disease.

Chapter 7: General discussion

Infection of mice with *S. typhimurium* results in systemic infection and the resultant disease resembles typhoid fever caused by *S. typhi* in humans (Wick et al., 2004). After oral ingestion and colonisation of the small intestine, *S. typhimurium* penetrates the intestinal epithelium and enters the Peyer's patches mainly through M cells or in some cases by an M cell-independent pathway. Subsequently, the bacteria spread into the draining mesenteric lymph nodes and disseminate via the bloodstream to the spleen and liver, where they replicate inside phagocytic cells (Niedergang et al., 2000).

The innate immune response to *S. typhimurium* infection involves recognition of bacterial components, such as LPS and flagellin through TLR (Aderem and Ulevitch, 2000), leading to phagocytosis of bacteria and activation of cells (Yrlid et al., 2000). In addition, bacterial components also induce a massive inflammatory response in the surrounding tissue. As a consequence, inflammatory mediators such as chemokines and cytokines are released, which function to activate and attract various cell types to the site of infection, amplifying the innate response.

Bacterial uptake and destruction by phagocytic cells facilitate host protection. Phagocytic cells, including macrophages (Fields et al., 1986; Wick et al., 1995), neutrophils (Yang et al., 2002) and DC (Yrlid et al., 2001a; Yrlid and Wick, 2002), are critical components of the innate immune response to *S. typhimurium* infection. The capacity of these phagocytic cell subpopulations to engulf GFP+ *S. typhimurium* in the spleen has been assessed (Yrlid et al.,

2001a). This study showed that neutrophils (CD11c-Gr-1+ cells) had the greatest capacity to associate with GFP+ *S. typhimurium in vivo*, followed by macrophages (CD11c-F4/80+ cells) and splenic DC (CD11c+MHCII+ cells), which had the lowest fraction of GFP+ cells. In addition, another study using GFP expressing *S. typhimurium* showed that all three splenic DC subsets associate with *S. typhimurium* (Yrlid and Wick, 2002). At the lowest dose of bacteria administered (10^8), a higher percentage of GFP+ cells was detected in the CD8α+ DC subset than in CD4+ and DN populations. Conversely, at a higher dose of bacteria (10^9), a similar percentage of GFP+ cells was detected in each subset (Yrlid and Wick, 2002). In our study, although we did not analyse neutrophils, we found that BMMac (CD11b+ CD11c- cells) had a greater capacity to internalise *S. typhimurium* than BMDC (CD11b+ CD11c+ cells) after high dose *in vitro* infection (10 M.O.I) (Figure 3.14), which is in agreement with above observations.

Upon phagocytosis, production of cytokines and NO are important mechanisms in combating *S. typhimurium* infection. Infection studies in mice using neutralising antibodies and knockout mice have shown that cytokines such as TNF-α, IFN-γ and IL-12 are important in the early stages of *Salmonella* infection and enhance host survival (Eckmann and Kagnoff, 2001). In our study, we found that BMMac produced greater amounts of TNF-α and to a lesser extent IL-12p40 than BMDC in response to *S. typhimurium* infection. Consistent with these findings, it has been demonstrated that cells producing TNF-α during the early stage of oral *Salmonella* infection are mainly neutrophils and macrophages rather than DC (Wick et al., 2004). Given the critical role of TNF-α

during *Salmonella* infection (Everest et al., 1998; Mastroeni et al., 1993), the production of large amounts of TNF-α by BMMac observed in our study suggests an important *in vivo* contribution of macrophages in controlling bacterial replication during the early stage of *Salmonella* infection.

Several IL-12 family cytokines have been identified. Among them, IL-12p70, consisting of covalently linked IL-12p40 and IL-12p35 subunits, is essential for protection against intracellular pathogens including *Salmonella*, and acts as by enhancing the induction of Th1 immune responses (Schuetze et al., 2005). Heterodimerisation of IL-12p40 with the p19 (a p35-related subunit) forms IL-23 (Oppmann et al., 2000), which has been suggested to be a critical factor for autoimmune inflammatory responses (Cua et al., 2003) and T cell proliferation (Oppmann et al., 2000). The third identified member of the IL-12 cytokine family is IL-27, which is a heterodimeric protein consisting of Epstein Barr virus induced protein 3 (EBI3, a p40 related subunit) and p28 (a p35 related subunit). The function of IL-27 is still in debate, but some studies show that IL-27 acts synergistically with IL-12p70 to play a role in initiation of a Th1 response (Takeda et al., 2003). Since the IL-12p40 subunit is a component of IL-12p70 and IL-23, IL-12p40 production cannot be directly related with the production of IL-12p70; additional signals are needed to facilitate the actual production of IL-12p70. In addition to this, a homodimer of p40, IL-12$(p40)_2$, also exists and has been suggested to act as an antagonist of IL-12p70 by competing for binding to the IL-12 receptor IL-12Rβ1 (Gillessen et al., 1995). Interestingly, a recent study has shown that the IL-12p40 subunit itself has a role in host resistance to *Salmonella* that is independent of IL-12p70 an IL-23 (Lehmann et al., 2001).

Therefore, although we have demonstrated that *Salmonella* infection could induce macrophages and DC to produce IL-12p40, the role of IL-12p40 during *Salmonella* infection appears to be complex. This issue could be further addressed by measuring and comparing the production of IL-12p40 and IL-12p70 in response to *Salmonella* infection in the future work.

Direct *ex vivo* analysis of intracellular cytokine production by defined cell populations from *Salmonella*-infected mice has revealed that NKT cells and TCR$\alpha\beta$ T cells are early sources of IFN-γ (Kirby et al., 2002). Surprisingly, macrophages were also reported to be efficient producers of IFN-γ during the early stage of primary infection with *S. typhimurium* (Kirby et al., 2002). However, we failed to detect any production of IFN-γ from macrophages in our study, which represents a discrepancy between our results and the other work. In this respect, it is worth noting that small number of contaminating CD8α+ T cells or NK cells can lead to the erroneous conclusion that macrophages are producing IFN-γ (Schleicher et al., 2005).

Another aspect of the innate immune response is represented by the recruitment of large numbers of cells to inflamed tissues, which is stimulated through the release of chemokines after infection. CXCL8/IL-8 is secreted by epithelial cells, attracting neutrophils that subsequently migrate through the epithelial layer into the intestinal lumen in response to *Salmonella* infection (Gewirtz et al., 2000; Gewirtz et al., 2001). Another chemokine, CCL20 is of particular importance in attracting cells such as immature DC to inflamed intestinal tissue in response to *Salmonella* infection (Izadpanah et al., 2001),

under these conditions, it appears that flagellin is the major factor responsible for stimulation of epithelial CCL20 production (Sierro et al., 2001).

DC and macrophages are also prominent producers of various chemokines upon stimulation (Foti et al., 1999; Sallusto et al., 1999). Kinetic studies have shown that DC produce inflammatory (such as CCL3, CCL4, CCL5 and CXCL8) and constitutive chemokines (including homeostatic and dual function chemokines such as CCL19 and CCL17) in a time-ordered fashion (Sallusto et al., 1999). At early time points (3-24h after stimulation) large amounts of inflammatory chemokines are produced. This declines at later time points (10-30h), when constitutive chemokines are selectively upregulated instead. A similar pattern of regulation of inflammatory chemokines (CCL3 and CCL5) upon DC maturation was observed in another study (Foti et al., 1999).

In contrast, activated macrophages also produce a similar spectrum of inflammatory chemokines, but do not produce constitutive chemokines (Sallusto et al., 1999). In addition, the production of inflammatory chemokines by macrophages is sustained, and the level of production is increased at later time points. These observations imply a differential role of DC and macrophages in recruiting various cell types. For DC, the production of inflammatory chemokines at early time points when DC are still in the peripheral tissues contributes to the recruitment of cells such as immature DC and their precursors, neutrophils and macrophages, which express the receptors for those chemokines. This recruitment is important to enhance and sustain antigen sampling. At later time points when DC have already reached the lymph nodes,

the production of constitutive chemokines, such as CCL19, may allow mature DC to attract naive T and B cells as well as maturing DC, which all express CCR7. Moreover, the production of CCL17 might favour the interaction of mature DC with distinct T cell subsets that express CCR4. This phase of recruitment functions to prime T cells and to initiate the acquired immune responses. Unlike DC, macrophages sustain the production of inflammatory chemokines but lack the ability to produce constitutive chemokines. This may function to continually recruit immature DC, neutrophils and macrophages to the site of inflammation in order to amplify the cascade of immune responses. Notably, not only the whole bacteria or LPS, but also cytokines such as TNF-α, generated upon infection, can induce DC and macrophages to produce inflammatory chemokines (Sallusto et al., 1999). Therefore, these observations, together with our results that macrophages produce more TNF-α than DC upon *Salmonella* infection, support the notion that macrophages may be more potent than DC in attracting newly recruited phagocytic cells to the site of infection.

DC and macrophages are also thought to contribute to systemic dissemination of bacteria during *Salmonella* infection. A previous study has shown that *S. typhimurium* is found in the bloodstream within CD18+ expressing phagocytes, including DC and tissue macrophages, as early as 15 minutes after oral inoculation (Vazquez-Torres et al., 1999). Interestingly, our study also showed that intracellular *S. typhimurium* could be transported to the spleen and liver via the trafficking of DC and macrophages, and was detected two days after i.p. adoptive transfer (Figure 4.6 and Figure 4.7). Hence, the transferred DC and macrophages are able to gain access to the bloodstream. Bloodstream

trafficking of *S. typhimurium* within the phagocytes may on the one hand facilitate antigen presentation in the spleen and LNs and the development of a systemic immune response, and on the other hand, provide a means for systemic dissemination of *S. typhimurium* throughout the body.

The innate immune system can restrict replication of *S. typhimurium* to a certain degree, but acquired immunity is required for effective control and eradication of bacteria. The fact that *Salmonella* has evolved an array of virulence systems to force its uptake by host cells, promote its intracellular survival and cause apoptosis of infected cells (Hansen-Wester and Hensel, 2001) makes acquired immunity particularly important for clearing of *Salmonella* infection.

A prerequisite for initiating acquired immune responses against *S. typhimurium* is the participation of DC. DC are characterised by their unique migratory capacity, first to the sites of infection and then to the draining LN, which is facilitated by a switch in the expression of chemokine receptors (Fleming et al., 2003; Ohl et al., 2004; Yanagawa and Onoe, 2003). Immature DC express receptors for inflammatory chemokines such as CCR1, CCR2, CCR5, CCR6 and CXCR1. These chemokines guide DC to inflammatory sites where antigen sampling can take place and maturation can be induced. The maturation process, which is triggered by microbial products or inflammatory cytokines, leads to down-regulation of receptors for inflammatory chemokines and up-regulation of receptors for constitutive chemokines such as CCR7. The expression of CCR7 may drive the maturing DC first towards the lymphatics and

then into T cell areas within the lymph nodes, where they encounter and stimulate naive T cells.

In our study, BMDC underwent a switch of expression of chemokine receptors upon infection with *S. typhimurium*. Examined as a representative receptor for inflammatory chemokines, CCR6 was shown to be downregulated, whereas CCR7 was greatly upregulated (Figure 5.3). Furthermore, we showed that this was a functional switch in chemokine responsiveness, demonstrated by an *in vitro* chemotaxis assay (Figure 5.8 and Figure 5.9). Loss of responsiveness to the CCR6 ligand, CCL20 may allow DC to escape from the peripheral tissues. Conversely, acquiring CCR7 expression was shown to be necessary for migration of *Salmonella* infected-DC to lymph nodes (Figure 5.13). In contrast, although BMMac showed a similar pattern in the switch of their chemokine receptors, CCR7 expression on *Salmonella*-infected macrophages was far less than on DC (Figure 5.5). This may be the reason why macrophages failed to migrate to SLO *in vivo* (Figure 4.2). Rather, these cells migrated to inflamed joints after adoptive transfer (Figure 4.12). This differential migration pattern of DC and macrophages implies different consequences of bacterial uptake by these two cell types: T cell priming by DC and transport of *Salmonella* to various peripheral tissues by macrophages.

Using recombinant *S. typhimurium* expressing T cell epitopes for infection, our study showed that both BMDC and BMMac could efficiently stimulate antigen experienced CD8+ T cells (Figure 6.1). However, only BMDC were able to activate naive T cells (Figure 6.2). More importantly, adoptive transfer of

recombinant *S. typhimurium* infected BMDC led to the stimulation of antigen specific naive T cells *in vivo* (figure 6.5). These results provide evidence that adoptive transfer of *S. typhimurium* infected-BMDC was an effective approach to deliver antigen *in vivo*. Due to the lack of an appropriate antigen model, we have not tested for the induction of MHC-II restricted CD4+ T cell responses by BMDC upon *S. typhimurium* infection. However, previous data has shown that BMDC can process *Salmonella* antigens not only for MHC-I presentation to CD8+ T cells, but also for MHC-II presentation to CD4+ T cells (Svensson et al., 1997b).

After specific immune responses are induced, the immune effectors are attracted to the sites of infection. At this stage, macrophages are also important effector cells for resolving infection with intracellular bacteria. Activation of macrophages by cytokines such as IFN-γ and TNF-α, leads to an induction of bactericidal mechanisms in macrophages (Gulig et al., 1997; Nauciel and Espinasse-Maes, 1992). These mechanisms include not only production of reactive oxygen and nitrogen intermediates but also improved handling of bacteria-containing phagosomes, rendering the bacteria accessible to lytic effector molecules from the lysosomes (Mittrucker and Kaufmann, 2000). *Salmonella* use an SPI-2 TTSS mediated mechanism to allow them to reside within the modified phagosomes, altering the trafficking of lysosomes to avoid fusion of phagosomes with lysosomes (Linehan and Holden, 2003). In addition, SPI-2 minimises contact of *Salmonella* phagosomes with vesicles rich in NADPH oxidase or iNOS, thus reducing exposure to reactive oxygen and nitrogen intermidiates (Linehan and Holden, 2003). Recently, a study has

shown that IFN-γ activated macrophages producing NO abrogate the intracellular survival advantage of *Salmonella* conferred by SPI-2, therefore enhancing the antimicrobial activity of macrophages (McCollister et al., 2005). These effects confirm the important role of cytokine-mediated activation of macrophages in the host response against *Salmonella* infection.

Taken together, the available data suggest that although macrophages and DC are both involved in anti-*Salmonella* immunity, the precise role that they play differs. Macrophages serve as a major contributor to the innate response and function primarily to control bacterial replication, provide the initial source of cytokines such as TNF-α and IL-12 and recruit new cells to the site of infection. In addition, macrophages also serve as an important effector of the acquired immune system and facilitate elimination of invading bacteria. In contrast, DC, serving as a critical link between the innate and acquire immune system and as the most potent APC, function primarily in triggering T-cell responses, being particularly crucial for naive T cell responses. This requires distinct migratory patterns of DC at different stages of infection, which is mediated by complex regulation of chemokine and chemokine receptor expression by infection-associated signals.

In addition to furthering our understanding of the distinct functions of macrophages and DC during *Salmonella* infection, our studies also have implications for the role of macrophage and DC in the pathogenesis of ReA.

Migration results showed that adoptively transferred macrophages, but not DC were detected in the inflamed joints of mice previously infected with C5 (TS) (Figure 4.12-4.13). CD18-expressing cells, representing cells of the monocyte-macrophage lineage and consisting of DC and/or tissue macrophages, have been demonstrated to carry *S. typhimurium* in the bloodstream of mice after oral inoculation (Vazquez-Torres et al., 1999). Our experimental data provide direct evidence for a pathway in which activated macrophages are able to traffic to peripheral tissues such as limbs from the peritoneal cavity; this occurred via the bloodstream, as cells also could be detected in the spleen and liver (data not shown).

Although this distinctive migration of macrophages was observed under conditions of induced inflammatory arthritis, parallels may exist for an antigen transportation role in ReA. ReA can occur following *Salmonella* infection (Mattila et al., 1998). In conjunction with this, the gastrointestinal tract is the largest reservoir of macrophages in the body (Smith et al., 1997), and macrophages may pick up *Salmonella* antigens from the original infection site (e.g. intestinal tract) and then gain access to the bloodstream as CD18+ cells. Alternatively blood borne monocytes can encounter *Salmonella* antigens in the bloodstream and differentiate into macrophages. In addition to this, 50% of synovial tissue cells are populated by macrophages recruited from peripheral blood (Gaston, 1998). This property of the synovium increases the likelihood that bacterial antigens within macrophages could gain access to joints from the blood stream. Whether migration of infected macrophages to sterile joints also occurs needs to be addressed in future work.

Another area for future study is the issue of the relevance of *Salmonella* antigen associated with transferred macrophages within the joints. Since, in our model, C5 (TS) was used to induce inflammatory arthritis and for macrophage infection, it was not possible to define the source of bacteria in the joints. To tackle this problem, inflammatory arthritis could be induced by other agents such as collagen (Nanki et al., 2004), and identification of bacteria transported by macrophages could be demonstrated by co-localisation of staining with a macrophage marker (e.g. F4/80) and a bacterial marker (e.g. LPS) in the inflamed joint tissue. This experiment is feasible because our previous work has shown these antibodies to efficiently identify macrophages or *S. typhimurium* even in paraffin sections of joint tissues.

In contrast, *S. typhimurium*-infected DC were not detected in the inflamed joints, but were detected in the adjacent LNs (Figure 4.13). This fits with the view that DC position themselves in SLO to stimulate T cells, a process that is enhanced by the increased surface expression of co-stimulatory molecules induced upon *Salmonella* infection. Furthermore, we observed HLA-B27-restricted activation of naive CD8+ or CD4+ T cells in LNs of mice receiving *Salmonella*-infected DC (Figure 6.5). In this respect, our results were in support of the notion that HLA-B27-reactive CD4+ T cells are functional *in vivo* and may play a pathogenic role in SpA (Boyle et al., 2001; Roddis et al., 2004).

Once in the joints, production of inflammatory cytokines such as TNF-α from activated macrophages is sufficient to cause a local inflammation. Various

inflammatory chemokines such as CCL5 (Volin et al., 1998) and CX_3CL1 (Ruth et al., 2001) produced by macrophages may attract more phagocytes such as macrophages and immature DC. In addition, effector T cells could also be attracted to the sites of inflammation. Production of Th1 cytokines, including TNF-α and IFN-γ and Th2 cytokines such as IL-4 by effector T cells would subsequently be produced at the tissue site. HLA-B27 could contribute to and alter this process through unconventional recognition by T cell subsets, including CD8+, CD4+ T cells and NK cells (Reveille and Arnett, 2005). An inappropriate cytokine milieu could be pathogenic rather than act to resolve the inflammation. Alternatively, HLA-B27 might also facilitate the persistence of intracellular bacteria (Laitio et al., 1997), increasing the duration and severity of the disease (Ekman et al., 2000).

Overall, it appears that macrophages are more likely than DC to play a role in transporting *Salmonella* antigens to the joints. In addition, macrophages might also be the initial source of inflammatory cytokines and chemokines that attract more immune cells into the joints. In contrast, DC serve as APCs to HLA-B27 restricted T cells, either in a conventional or non-conventional way. Therefore, in different ways, these two types of cells could potentially both contribute to the pathogenesis of ReA.

In our study, *S. typhimurium* infected-DC showed a significantly greater capacity to migrate to the spleen and MLNs compared to uninfected DC after i.p. transfer into mice. By using CCR7 deficient cells, we demonstrated that migration to the MLNs was CCR7-dependent, but to the spleen was CCR7-independent.

Since MLNs are in close proximity to the peritoneal cavity, these LNs were studied as potential draining LNs for peritoneal cavity in our study. This notion is supported by previous studies showing that radiolabelled fused aluminosilicate particles translocate mainly to MLNs and parathoracic LNs after being injected into the peritoneal cavity in dogs (Snipes et al., 1983). Similarly, it has been shown that the highest concentrations of gold colloid (198-Au) are found within the omentum and MLNs after i.p. injection into mice (Williams and Bradley, 1989). Moreover, labelled neutrophils were detected in the MLNs of mice after i.p. injection (Miyazaki et al., 2004). These studies demonstrated that MLNs can drain the peritoneal cavity. However, other studies have suggested that the principle draining LNs for the peritoneal cavity are the parathymic/mediastinal LNs; this was shown using transfer of diluted India ink (Geissmann et al., 2003; M. F. Abu-Hijleh, 1994). Thus, the issue of whether MLN could directly drain peritoneal cavity remains controversial. In our study, we did not assess whether *S. typhimurium* infected-DC could also be found in greater number in parathymic LNs, but this issue be worthy of investigation in the future work.

If DC did not enter MLNs through lymphatics draining the peritoneal cavity, by what alternative pathways could they gain access to MLNs? There are two possibilities: first, DC could access MLNs from the peritoneal cavity by direct movement through the lymphatic stomata, a mechanism that can be enhanced by NO (Li and Li, 2004). Second, DC could enter MLNs through HEV via the blood; the feasibility of this second option calls for further discussion. With the respect to the first step of such a pathway, the presence of transferred DC in

the spleen strongly implies that the cells had gained access to the bloodstream. The precise way in which DC did so is not clear, but it has been speculated that DC may first migrate to the draining lymph nodes and then enter the efferent lymph to gain access to the blood (Randolph et al., 2005). However, this idea would be challenged by a widely-accepted view that DC die after their arrival in LNs, and therefore can not leave LNs through efferent lymphatic vessels; this is supported by their absence in the efferent lymph (Banchereau and Steinman, 1998; Steinman, 1991). However, another study seems to counter this idea by showing that ~10% of the DCs that enter the LN exit by the efferent route (Dandie et al., 1994).

After entry into the bloodstream, DC could have gained entry to the LNs, such as MLNs and ILNs, through the HEV. Arguing against this idea is data showing that DC are unable to enter the LNs through blood after i.v administration (Austyn et al., 1988), and that only PDC precursors, but not myeloid DC precursors are able to transmigrate across HEVs directly from the bloodstream (Yoneyama et al., 2004). However, it has also been suggested that a pathway may exist whereby myeloid DC can migrate into LNs via HEV (Cavanagh and Von Andrian, 2002). Direct evidence to support this notion has been provided in an *in vivo* study showing that infection of mice with mouse mammary tumour virus induces a strong migration of MDC to the popliteal LNs via HEVs (Martin et al., 2002).

Although the exact pathways by which DC accessed MLNs is unknown, the migration of DC to MLNs was clearly dependent upon CCR7 expression (Figure

5.13). Migration of DC to the draining LNs through afferent lymph has been demonstrated to be CCR7-dependent (Ohl et al., 2004). In addition, a pathway involving DC migration to MLN via HEVs would likely be CCR7-dependent, in keeping with the expression of its ligands on HEVs (Gunn et al., 1998; Luther et al., 2000). At present, the dependence of CCR7 expression for direct DC movement through lymphatic stomata is not known.

Interestingly, our results showed that transferred DC localised mainly in the red pulp and marginal zone areas of the spleen (Figure 4.10 middle panel). Some cells, especially *S. typhimurium* infected DC migrated into the centre of white pulp (T cell zone) (Figure 4.10 lower panel). These results suggested the existence of a secondary migration of *S. typhimurium* infected-DC from red pulp to white pulp after they had arrived in the spleen. Although migration of DC to the spleen was not mediated by CCR7, this secondary migration of DC could potentially be affected by CCR7 owing to expression of CCR7 ligands within the white pulp (T cells zones), but not red pulp. A simple way to study this would be to investigate the localisation of CCR7 deficient DC within the spleen after transfer.

Other studies have also shown that CCR7 expression by DC is required for the entry of DC into lymphoid tissue under both steady state and inflammatory conditions (Martln-Fontecha et al., 2003; Ohl et al., 2004). However, it cannot be excluded that other signals in addition to CCR7 are also involved in controlling this migration pathway. In a recent study, it has been shown that CCR8 and its cognate ligand CCL1 are also involved in the migration of mouse

monocyte-derived DC to the draining LNs (Qu et al., 2004). In addition, mature DC also express other chemotactic receptors such as CXCR4, that potentially contribute to DC migration into lymphoid tissues (Sozzani, 2005). Finally, molecules other than chemokines and chemokine receptors, such as adhesion molecules, are crucially involved in the process of DC migration. For example, data have shown that mice defective in the β2 integrins (Xu et al., 2001) or α6 integrins (Price et al., 1997) display a reduction in the migration of cutaneous DC to the draining LNs. On the contrary, mice defective in JAM-A, an adhesion molecule expressed by DCs and the lymphatic endothelium, have an enhanced migration of skin DC to LNs (Cera et al., 2004). All these findings indicate that migration of DC is a multistep process involving multiple molecules.

DC-based vaccination has been used in immunotherapy, especially in the treatment of cancer. A DC vaccine, such as DC loaded with tumour associated antigen, has been used in clinical treatment of patients with cancer (Figdor et al., 2004). Currently, there are two general approaches to load antigens onto DC: the *in vivo* route and *ex vivo* route (Gilboa, 2004).

Several strategies to target antigens to DC *in vivo* have been described. For example, targeting of antigens to DC in mice through the DC specific cell-surface molecule DEC205, combined with an additional treatment such as a CD40-specific antibody to activate DC, has been shown to stimulate immunity (Bonifaz et al., 2002). Although, it is in theory an ideal solution to load tumour antigens onto a patient's own DC, its effectiveness could be compromised if DC function has been adversely affected in patients with cancer (Vicari et al., 2004).

Alternatively, strategies designed to load DC with antigens *ex vivo* have gained increasing attention due to the efficiency of such a process, and the ability to control the manipulation and generation of optimally differentiated immunostimulatory DC. In addition, for cancer treatment, this method could circumvent possible defects in patient DC (Vicari et al., 2004). Therefore, adoptive transfer of antigen loaded DC has been widely used in immunotherapy. Several clinical trials have demonstrated that immunisation with tumour antigen-pulsed DC could break tolerance against antigens expressed by tumour cells, and in some cases, generate appreciable clinical responses. Hence, this represents a promising method for the treatment of malignancies (Cerundolo et al., 2004; Engleman, 2003; Peng et al., 2005).

Our results have some implications for the practical application of this type of DC vaccination. Firstly, CCR7 expression can mediate DC migration from peripheral tissues to LNs, and the level of migration is associated with the extent of maturation, due to the higher expression of CCR7. This indicates that DC do not have to be directly injected into draining LNs, which may potentially destroy the architecture of the LNs, even though this approach is thought to avoid the need for DC migration into the LNs (Jonuleit et al., 2001). Instead, our results favour the idea that DC can be administered intradermally or intravenously, after which DC will migrate to the LNs to exert their antigen presentation role. Secondly, full activation of DC is required for the effective priming. Our *in vivo* study indicated that recombinant *S. typhimurium* infected DC showed a slightly greater stimulation of antigen specific CD8+ T cells than

peptide-pulsed DC (Figure 6.5). This is thought to be due to their lower expression of CCR7 and costimulatory molecules compared to bacteria-infected DC. These results are consistent with the current consensus that activation of DC is essential to induce immunity during immunotherapy (Tan and O'Neill, 2005).

Taken together, our study demonstrated that CCR7 mediated migration of DC to SLO is a prerequisite for induction of a therapeutic T cell response. In this respect, the results therefore contribute to further understanding of the biological mechanisms underlying DC-based immunotherapy.

Overall, the findings presented in this thesis broaden our knowledge about the contributions of macrophages and DC in anti-*Salmonella* immunity. In addition, the differential migration profiles of macrophages and DC upon *Salmonella* infection also shed light on their distinct roles in the pathogenesis of ReA. The presence of transferred macrophages in the joints provides direct evidence that transportation of bacteria by macrophages from one peripheral site to another could occur *in vivo*. Finally, this is the first report showing that the migration of DC to MLNs, but not to spleen, is mediated by CCR7 after i.p. injection. CCR7-mediated migration was followed by the induction of antigen specific T cell responses in LNs, providing information relevant to the application of DC vaccine-related immunotherapy.

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